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(54) Title: NUCLEAR TRANSFER FOR PRODUCTION OF TRANSGENIC ANIMAL EMBRYO

(57) Abstract

A method of generating a transgenic animal embryo which method includes providing a donor nucleus and a recipient cell; removing the nucleus for the recipient cell; introducing the donor nucleus into the enucleated cell to produce a couplet; and maintaining the couplet in a suitable medium for a period sufficient to allow the cell to recover a substantially normal shape. The method can be applied for porcine embryos and is exemplified with heterogenic expression of α1,3–galactosyl transferase.

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NUCLEAR TRANSFER FOR PRODUCTION OF TRANSGENIC ANIMAL EMBRYO

This invention relates to the generation of animals by nuclear transfer, including but not limited to the generation of transgenic animals.

Nuclear transfer is the replacement of the nucleus of one cell with that of another. The ability to produce live offspring by nuclear transfer is an objective which has been sought for some time by animal breeders. The ability to produce cloned offspring in such a manner would enable the production of large numbers of identical offspring and the ability to genetically modify and/or select cell populations of the required genotype (e.g. sex or transgenic) prior to embryo reconstruction.

Whilst nuclear transfer has been described in some animals, the procedures used are often inefficient and have not yet been successfully applied to many species. Also, there are no examples of gene activity being modified in a large animal using nuclear transfer.

Applicants have discovered that, in relation to porcine embryos in particular, cloning techniques known in the prior art for ovine and bovine animals (see for example WO97/07669 and WO95/17500) cannot be applied to porcine animals.

It is an object of the present invention to overcome, or at least alleviate, one or more of the difficulties or deficiencies associated with the prior art.

In a first aspect of the present invention there is provided a method of generating an animal embryo which method includes

providing

a donor nucleus, and

a recipient cell;

removing the nucleus from the recipient cell;

introducing the donor nucleus into the enucleated cell to produce a ~~reconstituted cell~~ complet; and

maintaining the ~~reconstituted cell~~ complet in a suitable medium for a period

As used herein a "couplet" is a complex comprising an enucleated recipient cell and a donor nucleus, wherein the donor nucleus is not completely within the plasma membrane of the recipient cell. In preferred embodiments, the recipient cell is an oocyte and the donor nucleus is positioned between the oocyte membrane and the zona pellucida.

A "donor nucleus" is the genetic material of a donor cell, whether or not bounded by one or more membrane. Oocyte nuclei are preferably the metaphase plate of the oocyte.

A "recipient cell" is a cell which obtains a donor nucleus.

sufficient to allow the cell to recover a substantially normal shape.

Applicants have discovered that the number of viable embryos produced may be significantly increased by permitting the ~~reconstituted cell~~ couplet to be maintained in a quiescent state for a period sufficient to allow the cell to recover a substantially normal, e.g. generally circular, shape.

Whilst we do not wish to be restricted by theory, it is postulated that the quiescent period permits the cell to return to a more normal state after which cell fusion may proceed more efficiently.

The ~~reconstituted cell~~ couplet may be maintained in a suitable medium for a period of approximately 3 to 8 hours, preferably approximately 4.5 to 6 hours.

It is desirable, however, that the quiescent period end before any, or any substantive division, ensues.

In a preferred embodiment of the present invention, the method may include the preliminary step of

subjecting the recipient cell to an activation step; and

subsequently removing the nucleus from the activated cell.

Applicants have found, in this preferred embodiment, improved results where a preliminary activation step is undertaken.

Preferably the ~~reconstituted cell~~ couplet is subsequently subjected to cell fusion.

Where the preferred preliminary activation step is not utilised the ~~reconstituted cell~~ couplet may be subjected to a cell fusion/activation step. For example, where electrical pulses are utilised for cell fusion, the voltage may be selected to simultaneously initiate activation.

In a further aspect of the present invention there is provided a method of generating a transgenic animal embryo said method including

providing

a donor nucleus which has been genetically modified to eliminate an undesirable activity or to provide for a desirable activity, and

a recipient cell;

transferring the donor nucleus to the recipient cell to produce a complet ~~reconstituted cell~~; and

generating a transgenic animal embryo from said complet ~~reconstituted cell~~.

The animal embryo may be of any type, and includes bird, fish, reptile and mammalian (including ungulate and primate) embryos including human embryos, e.g. murine, bovine, ovine or porcine embryos. Preferably, the animal embryo is a porcine embryo, bovine embryo, murine embryo or human embryo.

Preferably the transfer step includes

removing the nucleus from the recipient cell;

introducing the donor nucleus into the enucleated cell to produce a complet ~~reconstituted cell~~; and

maintaining the complet ~~reconstituted cell~~ in a suitable medium for a period sufficient to allow the cytoplasm to recover a substantially normal shape.

The donor nucleus may be of any suitable type and from any suitable species. The donor nucleus may be contained in a karyoplast or cell. The donor nucleus may be of embryonic, embryonal tumor, foetal or adult origin. Donor nuclei may be prepared by removing the nucleus and a portion of the cytoplasm and plasma membrane surrounding it from early pre-implantation stage embryos (for example zygotes, 4- to 16- cell embryos) for example using microsurgery. When nuclei from more advanced embryonic cells are used the whole blastomere may be transferred to the recipient cytoplasm. Embryonic or foetal fibroblasts may be used. Embryonic stem (ES) cells [isolated from inner cell mass (ICM) cells, embryonic disc (ED) cells or primordial germ cells (PGC)] may be used. A cell line derived from an embryonal tumor may be used (eg. embryonal carcinoma (EC) or yolk sac tumor cells). Adult cells such as fibroblasts may also be used. In this case the whole cell may be fused to the recipient cytoplasm.

The recipient cell may be of any suitable type and from any suitable species. Cytoplasts may be prepared from *in vivo* or *in vitro* produced oocytes. Cytoplasts may be prepared from oocytes arrested in the second metaphase of meiotic maturation (MII oocytes). Other sources of cytoplasts include zygotes, fertilised oocytes, and 2-cell blastomeres.

Cytoplast preparation involves the removal of the nucleus in a process referred to as enucleation. The nucleus may be removed by microsurgery. This may involve the removal of pronuclei or metaphase plate and surrounding cytoplasm from zygotes or oocytes, for example by aspiration or embryo bisection. Such manipulation may follow incubation of the zygotes or oocytes in a microfilament inhibitor, for example cytochalasin B (Sigma Cell Culture, Sigma-Aldrich Pty. Ltd.), that relaxes the cytoskeleton and allows the removal of a portion of membrane enclosed cytoplasm containing the pronuclei or metaphase plate. Alternatively, nonphysical approaches such as inactivation of the chromosomes by UV (chemical) or laser irradiation may be used.

The donor nucleus may be transferred to the recipient cell by any suitable method. Such methods include, but are not limited to, microsurgical injection, and cell fusion mediated by for example electrical pulses (electrofusion), chemical reagents such as polyethyleneglycol or the use of inactivated virus such as Sendai virus.

Preferably the donor nucleus is introduced under the zona pellucida.

In a further preferred aspect the donor nucleus is introduced into the enucleated cell substantially immediately after enucleation.

More preferably the nucleus is removed from the recipient cell via an incision site and the donor nucleus is introduced into the enucleated cell through the same incision site.

Cytoplast volume may be increased by fusing together zona pellucida free cytoplasts before, after or at approximately the same time as donor nucleus

In another aspect, the invention provides a method for generating an animal embryo, the method comprising providing a donor nucleus and a recipient cell; removing a nuclear from the recipient cell; maintaining the recipient cell in a suitable medium for a period sufficient to allow the cell to recover a substantially normal shape; and transferring the donor nucleus to the recipient cell by direct injection to produce a reconstituted cell.

fusion.

An animal embryo may be generated from the ~~reconstituted cell~~ reconstituted cell or complet by any suitable method. Embryonic development may be initially *in vitro* and subsequently in a surrogate. Thus, the ~~reconstituted cell~~ reconstituted cell or complet may be initially cultured *in vitro* to produce an embryo and then the embryo may be transferred to a surrogate for subsequent development into an animal. *In vitro* culture of the ~~reconstituted cells~~ reconstituted cell or complet may be in any suitable medium.

Activation occurs during fertilisation when the penetrating sperm triggers the resumption of meiosis. Activation is characterised by calcium oscillation, release of cortical granules, extrusion of the second polar body, pronuclear formation and ultimately cleavage. The reconstituted cell or complet may be treated with, for example, ethanol, calcium ionophore or electrical stimulation to induce activation. Activation is performed prior to or after transfer of the donor nuclei.

In a preferred embodiment of this aspect of the present invention, the donor nucleus may be from an embryo that is itself the product of nuclear transplantation. This is known as serial nuclear transfer.

Serial nuclear transfer may improve the capacity of differentiated nuclei to direct normal development. Whilst applicant does not wish to be restricted by theory, serial nuclear transfer is postulated to improve the developmental capacity of transplanted nuclei by allowing specific molecular components in the oocyte to assist in chromatin remodelling that is essential for nuclear reprogramming. Serial nuclear transfer is not restricted to a singular event but may be initiated on more than one occasion to sequentially improve conditions for chromatin remodelling, nuclear reprogramming and embryonic development

The donor nucleus and recipient cytoplasm which are used in the method of the present invention may be of any suitable origin. Preferably, they are of porcine, bovine, murine or human origin. More preferably, they are from a MHC (SLA) miniature swine because isogenic embryos and cell lines may be derived from such animals (see below).

The method of the present invention may be used to generate transgenic animals. For example, a new gene may be expressed and/or an existing gene may be deleted in the transgenic animal. The addition of new genes is technically less demanding than the deletion of existing genes.

As used in this specification the term "transgenic", in relation to animals and all other species, should not be taken to be limited to referring to animals containing in their germ line one or more genes from another species, although many transgenic animals will contain such a gene or genes. Rather, the term refers more broadly to any animal whose germ line has been the subject of technical intervention by recombinant DNA technology. So, for example, an animal in whose germ line an endogenous gene has been deleted or modified (either by modifying the gene product or pattern of expression) is a transgenic animal for the purposes of this invention, as much as an animal to whose germ line an exogenous DNA sequence has been added.

The donor nucleus may be genetically modified by modifying, deleting or adding one or more genes. The gene(s) to be modified, deleted or added may be of any suitable type. In a particularly preferred embodiment of this aspect of the invention, the α1,3-galactosyltransferase gene may be modified in transgenic swine. This gene is non-functional in humans and so it is no longer expressed. It does, however, still function in swine where its role is to add a specific sugar configuration to protein backbones. This glycosylation pattern is highly antigenic and humans carry high levels of antibodies to it. Swine antigens to which humans react strongly are described as xenoantigens and the reactivity of humans towards swine antigens is described as xenoantigenicity. The net result is the immediate rejection of transplanted swine tissue in human recipients. Inactivation, through gene modification, of the swine α1,3-galactosyltransferase activity may make swine organs, tissues, or cells less susceptible to immune recognition following transplantation of said organs, tissues or cells into humans.

The process of modifying a gene may involve the introduction of one or more mutations in both copies of the target gene. Suitable cells may take up the mutation(s) and then be used to generate an animal. One copy of the gene may

be disrupted in the cell and the resultant heterozygous animals bred with each other until one with both copies of the gene mutated is found. Alternatively, both copies of the gene may be modified *in vitro*.

To target an endogenous gene rather than introduce random mutations, a DNA construct (transgene) including a nucleic acid sequence which is substantially isogenic to at least one or more portions of the target gene except for the introduction of the one or more mutations may, be used.

The targeting DNA may comprise a sequence in which the desired sequence modifications are flanked by DNA substantially isogenic with a corresponding target sequence in the genome to be modified. The substantially isogenic sequence is preferably at least about 97-98% identical with the corresponding target sequence (except for the desired sequence modifications), more preferably at least about 99.0-99.5% identical, most preferably about 99.6% to 99.9% identical. The targeting DNA and the target DNA preferably share stretches of DNA at least about 75 base pairs that are perfectly identical, more preferably at least about 150 base pairs that are perfectly identical, even more preferably at least about 500 base pairs that are perfectly identical. Accordingly, it is preferable to use targeting DNA derived from cells as closely related as possible to the cell being targeted; more preferably, the targeting DNA is derived from cells of the same haplotype as the cells being targeted. Most preferably, the targeting DNA is derived from cells of the same individual (or animal) as the cells being targeted. Preferably, the targeting DNA sequence comprises at least about 100-200 base pairs of substantially isogenic DNA, more preferably at least about 300-1000 base pairs of substantially isogenic DNA, even more preferably at least 1000-15000 base pairs of substantially isogenic DNA.

As used herein, the term isogenic or substantially isogenic DNA refers to DNA having a sequence that is identical with or nearly identical with a reference DNA sequence. Indication that two sequences are isogenic is that they will hybridise with each other under the most stringent hybridisation conditions (see e.g., Sambrook J., et al., 1989); and will not exhibit sequence polymorphism (i.e. they will not have different sites for cleavage by restriction endonucleases). The

term "substantially isogenic" refers to DNA that is at least about 97-99% identical with the reference DNA sequence, and preferably at least about 99.5-99.9% identical with the reference DNA sequence and in certain cases 100% identical with the reference DNA sequence. Indications that two sequences are substantially isogenic is that they will still hybridise with each other under the most stringent conditions (see Sambrook, J., et al., 1989) and that they will only rarely exhibit restriction fragment length polymorphism (RFLP) or sequence polymorphism (relative to the number that would be statistically expected for sequences of their particular length which share at least about 97-98% sequence identity). In general, a targeting DNA sequence and a host cell sequence are compared over a window of at least about 75 consecutive nucleotides. DNA sequences compared between individuals of a highly inbred strain, such as the MHC inbred miniswine, are generally considered to be substantially isogenic even if detailed DNA sequence information is not available, if the sequence do not exhibit sequence polymorphisms by RFLP analysis.

Thus, the donor nucleus may be genetically modified by modifying an endogenous gene in the donor nucleus. The endogenous gene may be modified by introducing into said donor nucleus a DNA construct including a nucleic acid sequence which is substantially isogenic to at least one or more portions of the endogenous gene and includes one or more mutations, such that there is homologous recombination between the DNA construct and the endogenous gene.

In a further aspect of the present invention there is provided a DNA construct for modifying an endogenous gene in an animal nucleus, said DNA construct including a nucleic acid sequence which is substantially isogenic to at least one or more portions of the endogenous gene and includes one or more mutations.

In a preferred embodiment of this aspect of the invention the endogenous gene is a swine, bovine, ovine, caprine or murine gene, more preferably a swine α1,3-galactosyltransferase gene. In a particularly preferred embodiment of this aspect of the invention, the DNA construct is pGallaway, as hereinafter described

which suggests that any other standard commercially available gene delivery agent having an efficiency of at least 20% may be used according to the present invention.

[0061] Constructs to target the Rosa 26 locus, RNA pol II and GAPDH loci have been developed to show that any cloned loci of interest may be targeted. Several variations of such plasmids have been used. Either promoter-containing or promoter-less constructs with or without splice donor or acceptor sites may be used. Constructs with IRES sites or floxed gene products may be made using methods that are well described and readily obtainable by one skilled in the art. A detailed review of vectors and constructs used for homologous recombination is described in (Court et al., 2002, Copeland et al., 2001) and examples of some variants of vectors are described herein (FIG. 2.)

[0062] A vector may be promoter-less without an enhancer to be integrated downstream of an endogenous enhancer (*e.g.*, Rosa 26). According to the present invention, the vector may be a construct with an additional enhancer element that allows exogenous control of gene expression in addition to that provided by an endogenous enhancer as in the promoter-less vector. Promoters including, but not limited to CMV, PGK, prion proteins or any promoter suitable for driving expression in progenitor cell populations, may be integrated upstream of an endogenous gene, for example, one encoding GDNF.

[0063] A vector may be a construct with either a splice donor or splice acceptor site to allow expression following integration into specific regions of the targeted locus. A vector may be a construct with an IRES site to allow efficient expression of the desired protein following integration into a specific region of the endogenous gene. Further, according to the present invention, a suitable vector may be any variation of such constructs. Examples of such recombination are shown in Figures 3-6.

Example III

[0064] A vector was designed for homologous recombination. To construct a sequence that targeted the mouse Polr2a locus, IRES-neo sequences were cloned into the 3′ non-coding sequence (flanking exon 28) of the mouse Polr2a locus (FIG. 15) (SEQ ID. NO:1).

or a functionally equivalent plasmid.

By a functionally equivalent plasmid, we mean a plasmid capable of performing substantially the same function in substantially the same manner as the plasmid in question. Without limiting the generality of the foregoing, other plasmid backbones are contemplated. Also, plasmids containing other portions of the swine α1,3-galactosyltransferase gene and other mutations to that gene are contemplated.

The introduction of new genetic material and the subsequent selection of cells harbouring the desired targeted integration requires expansion and clonal selection of each founder transgenic cell. A limitation to applying this process in nuclear transplantation programs is the number of cell divisions which the transfected cell must undergo to provide sufficient material for molecular analysis of each transgenic colony and subsequent supply of nuclei for transfer. The great majority of cells suitable for *in vitro* genetic modification and subsequent nuclear transfer have limited *in vitro* propagation capacity. It is therefore desirable to utilise transfection and selection systems which generate and/or identify correctly targeted clones at high efficiency and with limited requirement for *in vitro* propagation.

A particularly efficient approach to selecting for correctly targeted clones is to use IRES gene trap targeting vectors, as described in Australian Patent 678234, the entire disclosure of which is incorporated herein by reference. The IRES gene trap targeting vector may be selected from IRES-neo, IRES-lacZ, (TAA_3) IRES-lacZ, (TAA_3) IRES-lacZ lox neo-tk lox, (TAG_3) IRES-lacZ/mcIneo, SA lacZ-IRES neo, SA (TAA_3) IRES-nuclear lacZ, SA (TAA_3) IRES-nuclear lacZ lox Gprt lox, IRES-βgeo, (TAA_3) IRES-βgeo, SA IRES-βgeo SA Optimised IRES-βgeo, IRES-nuclear βgeo, SA IRES-nuclear βgeo, SA (TAA_3) IRES-nuclear βgeo, SA Optimised IRES-nuclear βgeo, IRES-zeo, SA IRES-zeo, IRES-hph, SA IRES-hph, IRES-hph-tk, IRES-bsd, SA IRES-bsd, IRES-puro. IRES gene trap targeting vectors provide a significant enhancement in gene targeting efficiency by eliminating a large proportion of random integration events. IRES gene trap

targeting vectors rely upon functional integration into an actively transcribed gene (such as the target gene) for expression of the selectable marker. Random integrations into non-transcribed regions of the genome are not selected.

Accordingly in a further aspect of the present invention there is provided a DNA construct for modifying an endogenous gene in an animal nucleus, said DNA construct including

a nucleic acid sequence which is substantially isogenic to at least one or more portions of the endogenous gene and includes one or more mutations, and a selectable marker,

such that functional integration of said DNA construct into said endogenous gene results in expression of the selectable marker.

In a preferred embodiment of this aspect of the invention the endogenous gene is a swine, bovine, ovine, caprine or murine gene, more preferably a swine α1,3-galactosyltransferase gene. In a particularly preferred embodiment of this aspect of the invention, the DNA construct is pBERT 1, as hereinafter described or a functionally equivalent plasmid.

In a preferred embodiment, it may be desirable to remove the selectable marker cassette from the targeted locus to eliminate expression of the eg. antibiotic resistance gene. One approach is to flank the IRES selectable marker cassette with suitable DNA sequences which act as recombination sites following the addition of a suitable site-specific recombinase. One example of a suitable recombinase site is the lox site which is specific for the Cre recombinase protein. Another example of a suitable recombinase is the FLP/FRT recombinase system (O'Gorman, S., et al., 1991).

Accordingly in a further aspect of the present invention there is provided a DNA construct for modifying an endogenous gene in an animal nucleus, said DNA construct including

a nucleic acid sequence which is substantially isogenic to at least one or

more portions of the endogenous gene and includes one or more mutations

a selectable marker, and

recombination sites flanking said selectable marker,

such that functional integration of said DNA construct into said endogenous gene results in expression of the selectable marker and addition of a recombinase results in removal of the selectable marker by recombination at the recombination sites.

In a preferred embodiment of this aspect of the invention the endogenous gene is a swine or murine more preferably a swine α1,3-galactosyltransferase gene. In a particularly preferred embodiment of this aspect of the invention, the DNA construct is pBERT 11, as hereinafter described or a functionally equivalent plasmid.

High efficiency gene targeting and selection has a significant advantage in that suitably stringent selection systems, such as the IRES gene trap targeting vectors, can eliminate the need for biochemical analysis of clonal cell lines. In this instance, individual nuclei from a pool of uncharacterised transgenic cells should generate offspring of the desired phenotype at a ratio equivalent to the selected pool. The elimination of clonal selection may be particularly useful where only limited *in vitro* propagation is desirable or possible. One such instance includes the culture of embryonic nuclei for nuclear transfer. Embryonic nuclei are more efficient than latter stage somatic cells for generating live born offspring by nuclear transfer. However, totipotential embryonic cells can not be cultured for extended periods for any other species than mice. Nuclear recycling of embryonic nuclei (see following) provides an opportunity to maintain, expand and genetically manipulate multipotential cells from animals *in vitro*.

The DNA constructs according to this aspect of the invention may be engineered in bacteria and then introduced into the cells. The transgenes may be introduced into the cells by any suitable method. Preferred methods include direct injection, electroporation, liposomes or calcium phosphate precipitation. Direct injection is the preferred method for embryonic cells while electroporation is more suitable for embryonic fibroblast and embryonic stem cell cultures.

Whilst applicant does not wish to be restricted by theory, it is thought that regions of substantially isogenic DNA either side of the mutation drag the transgene to the target site where it recombines and introduces the mutation. It is further thought that the main contributing factor for increasing the efficiency of introducing a specific mutation in a given gene is the degree of similarity between the target DNA and the introduced DNA. Thus, it is preferred that the DNA is isogenic (genetically identical) not allogenic (genetically dissimilar) at the genetic locus that is to be targeted.

In a further aspect of the present invention there is provided an animal embryo or transgenic animal embryo produced by the methods of the present invention. Preferably the animal embryo or transgenic animal embryo is a porcine, murine, ovine, bovine, caprine or human embryo.

In a further aspect of the present invention there is provided a reconstituted animal cell or modified reconstituted animal cell produced by the methods of the present invention. Preferably the reconstituted animal cell or modified reconstituted animal cell is a porcine, murine, ovine, bovine, caprine or human cell.

In a still further aspect of the present invention there is provided an animal or transgenic animal produced by the methods of the present invention. Preferably the animal or transgenic animal is a porcine, murine, ovine, bovine, caprine or human animal, more preferably a MHC(SLA) miniature swine.

The present invention will now be more fully described with reference to the accompanying Examples and drawings. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

In the Figures:

Figure 1A shows an overview of a nuclear transfer procedure according to the present invention in which activation occurs prior to enucleation which is then

followed by a quiescent period of, e.g. 6 hours and then when appropriate fusion.

Figure 1B shows an overview of a nuclear transfer procedure according to the present invention in which recipient cells are enucleated, subjected to a quiescent period of, e.g. 6 hours and then activation or combined fusion/activation.

Figure 2 summarises the steps taken for construction of the pGallaway targeting vector, deposited in American Type Culture Collection, ATCC Accession No.

Figure 3 is a map of pBERT.

Figure 4 is a map of the linker referred to in Example 3.

Figure 5 shows the modifications to intron 8 and exon 9.

Figure 6 shows the genomic organisation of the swine α1,3-galactosyltransferase gene following a homologous recombination event between the chromosomal locus and the pGallaway vector.

Figure 7 shows the strategy used to identify mutant transfectants.

Figure 8 shows a PCR analysis of the transfectants obtained in Example 3.

Figure 9 shows the results of Ase 1 digestion of mutant PCR products.

Figure 10 is a map of pBERT 1.

Figure 11 is a map of pBERT 11.

Figure 12 shows transgenic animals generated by nuclear transfer.

Figure 13A shows an RT-PCR analysis of the transfectants obtained in Example 5.

Figure 13B shows the results of an RT-PCR analysis of the transfectants

obtained in Example 5 after Ase 1 digestion.

Figure 14 shows a strategy developed for screening for targeted colonies referred to in Example 12.

EXAMPLE 1

Cytoplast Preparation

Cytoplasts were prepared from either *in vivo* or *in vitro* produced oocytes collected from both outbred and MHC (SLA) inbred miniature swine. Whilst applicant does not wish to be restricted by theory, it is thought that the use of the MHC (SLA) inbred miniature swine oocytes as a source of cytoplasts will be beneficial in reducing mitochondrial DNA chimaerism and extraneous agents present in the cytoplast.

***In vivo* oocyte production**

A number of systems have been developed to synchronise and superovulate swine to obtain a large number of oocytes required for cytoplast preparation. These systems can be used in both outbred and MHC (SLA) miniature swine. Synchronisation of the estrous cycle was achieved by the following treatments: 1. administration of synthetic progesterone (eg. Regumate, Altrenogest, Hoechst-Roussel); 2. prostaglandin or prostaglandin analogue injection of a pregnant swine; 3. removal of offspring at weaning. Natural cycling swine was also used. Superovulation with follicle stimulating hormone (FSH) was used to increase the number of oocytes obtained from each ovary. The FSH activity of pregnant mares serum gonadotrophin (PMSG, Folligen, Intervet (Aust) Pty. Ltd. or Pregnecol, Horizon Technology Pty. Ltd. 750 -1500 IU) is preferred. Ovulation time was controlled with the injection of human chorionic gonadotrophin (hCG (Chorulon, Intervet (Aust) Pty. Ltd., 500 IU). Unfertilized metaphase 11 oocytes were surgically collected 2-16 hours after ovulation.

In vitro oocyte production

Pigs

Ovaries were collected from slaughtered gilts and transported to the laboratory at 37°C in Saline (0.9% Sodium Chloride) (HSA, Baxter AHB1324) supplemented with 10µl/ml penicillin-streptomycin solution (contains 5,000 iu/ml penicillin G sodium and 5,000µg/ml streptomycin sulfate, Gibco BRL, Life Technologies).

Oocyte cumulus complexes (OCC) were aspirated from follicles 2-6mm in diameter. OCC with uniform cytoplasm and compact cumulus cell mass were collected, rinsed in Dulbecco's phosphate buffered saline (Gibco BRL, Life Technologies Inc.) supplemented with 0.3% bovine serum albumin (ICN Biomedicals Inc.) and cultured in one of the following media:

Medium 1 Medium 199 (Sigma Cell Culture, Sigma–Aldrich Pty. Ltd.)

Medium 2 Modified Whittens medium (Whitten, W.K. and Bigger, J.D., 1968, Funahashi, H *et al.*, 1994b, 1996)

Medium 3 Modified G1 medium

A summary of porcine oocytes matured in the various media is shown in Table 1.

TABLE 1

In Vitro maturation of porcine oocytes

Medium	No. of oocytes examined	No. of oocytes with first polar body (%)
M199	352	220 (62.5)
Whittens	330	205 (62.1)
G1	383	150 (39.2)
Waymouth	275	71 (25.8)

OCC were cultured in microdrops or Nunc 4-well tissue culture plates of one of the above oocyte maturation medium or any other suitable medium overlaid with mineral oil (Sigma Chemical Company, Sigma-Aldrich Pty. Ltd.), at 38.5°C under 5%CO_2 in air for 48-50 hours. Each medium was supplemented with 10% Estrus cow serum (Day 0 to 5 after standing heat) and 0.01 units/ml Follicle Stimulating Hormone (from porcine pituitary, Sigma-Aldrich Pty. Ltd.) The benefits of adding of Estrus cow serum is shown in Table 2.

TABLE 2

Effects of different sera on the *in vitro* maturation of porcine oocytes

Sera	No. of oocytes examined	No. of oocytes with first polar body (%)
ECS	1187	728 (61.3)
FBS	271	86(31.7)

*ECS, Estrus Cow Serum; FBS, Fetal Bovine Serum

** Basic medium, TCM 199; Maturation time 48-52 hrs

Cattle

In vitro oocytes were obtained in an identical manner to porcine *in vitro* oocytes. Maturation was also in identical media, over a period of 22 hrs.

Microsurgical Enucleation or Bisection of nuclear material

Recipient cytoplast were prepared by removal of metaphase chromosomes from mature metaphase II (MII) oocytes, or both pronuclei (PN) from in vivo fertilised oocytes for the first and second round of nuclear transfer respectively.

Enucleation by microsurgery

Prior to enucleation the oocytes were denuded of cumulus cells by gentle pipetting after 2 minutes vortexing in 0.1% hyaluronidase (Sigma Cell Culture , Sigma-Aldrich Pty. Ltd., 300 iu/ml in Hepes buffered M199 or H-G1 (or other short term handling medium) and incubated in the UV fluorescent dye Hoechst 33342 (Sigma Cell Culture, Sigma-Aldrich Pty. Ltd. 5µg/ml) for approximately 10 minutes at 38.5^{0}C followed by washing in Hepes (Sigma Cell Culture, Sigma-Aldrich Pty. Ltd. or Gibco BRL, Life Technologies Inc.) buffered M199, G1 or other medium (listed above). The enucleation was performed in a 20 µl drop of Hepes buffered medium (M199 or G1) containing the cytoskeletal inhibitor, cytochalsin B (Sigma

Cell Culture, Sigma-Aldrich Pty. Ltd. 1.0 - 7.5 µg/ml). Using the micromanipulation pipettes oocytes were orientated so that the first polar body (PB) was clearly visible in the 12 o'clock position when the bevel of the enucleation pipette was upward facing. Once the oocyte was secured, the enucleation pipette was carefully inserted into the oocyte. The alignment of the holding and enucleation pipette with respect to the oocyte, zona pellucida and oolemma (cytoplasmic membrane) was important. Failure to have both pipettes and oocyte aligned resulted in lysis of the oocyte. Having penetrated the zona and oolemma, the enucleation pipette was positioned just below the first polar body. Gentle suction was applied into the enucleation pipette to remove a proportion (approximately 1/3 to 1/6) of the oocytes cytoplasm and the first polar body. Successful enucleation was confirmed by the detection of the metaphase plate in the removed cytoplasm and first polar body by exposing the enucleation pipette to UV light. Care was taken to avoid exposure of the enucleated oocyte to UV. In mouse experiments the first polar body was rarely removed as it is undergoing degeneration at this particular time. This enucleation procedure was performed at room temperature.

If the polar body was not observed prior to enucleation the oocyte was exposed to UV (<15 seconds) to allow for visualisation and orientation of the metaphase II plate. Following enucleation the oocyte and confirmation of enucleation was identical to the methods described above.

Cytoplast from the second round of nuclear transfer were prepared from in vivo sourced zygotes. Donor female gilts or swine were synchronised and superovulated as described above. Females were mated with males twice, 24 hours and 32 hours after hCG (Chorulon, Intervet (Aust) Pty. Ltd.) injection. Zygotes could also produced by *in vitro* fertilisation *of in vitro* matured oocytes.

Fertilised oocytes have two pronuclei: male and female pronucleus(PN), which appear 4-8 hours after fertilisation. The developing pronuclei are normally obscured by the high content of lipids present in swine embryos. Visualisation of the PN was achieved by centrifugation (6-12 min. 8,000-15,000g). Centrifugation stratifies the lipids and allowed both PN to be easily removed by enucleation techniques. Cumulus cells still adhered to the zona pellucida were removed by

incubation in hyaluronidase (300iu/ml) for approximately five minutes.

Cytoplasts for the purpose of serial nuclear transfer were prepared from zygotes 44-50 hours after hCG (Chorulon, Intervet (Aust) Pty. Ltd.) administration. Fertilised oocytes were incubated in cytochalasin B (Sigma-Aldrich Pty Ltd) in a manner similar to that described for MII oocyte enucleation, both PN were removed by microsurgery. No DNA staining was required to confirm the removal of PN since they were clearly visible in the enucleation pipette.

Enucleation by bisection

In vivo or *in vitro* matured oocytes were used for enucleation by bisection. The oocytes were denuded of cumulus cells by gentle pipetting or digestion in hyaluronidase (Sigma Cell Culture, Sigma-Aldrich Pty. Ltd., 300 IU/ml in H-G1 medium or other short term handling medium) and stained in UV fluorescent DNA dye Hoechst 33342 (Sigma Cell Culture, Sigma-Aldrich Pty. Ltd., 5 µg/ml) for approximately 10 minutes at 39°C. Metaphase II oocytes were selected . The zone pellucida was partially dissolved by a short incubation in 0.1% pronase (Sigma Cell Culture, Sigma-Aldrich Pty. Ltd.) and removed by a gentle passage through a narrow pippette. The oocytes were incubated 15 min in H-G1 or other short term medium with cytochalsin B (Sigma Cell Culture, Sigma-Aldrich Pty. Ltd.) (1.0 - 7.5 µg/ml) and enucleated by bisection into two halves (Tarkowski, 1977). The nucleus was likely to be located in close proximity to the first polar body. Phytohemagglutinin was used to stick the polar body to the cytoplasm membrane. The enucleated halves, identified under UV illumination by the absence of chromatin stained with Hoechst (Sigma Cell Culture, Sigma-Aldrich Pty. Ltd.), were washed and stored in H-G1 medium or other short term medium until used for fusion (46-50 hours after the onset of oocyte maturation).

EXAMPLE 2

Karyoplast preparation

Karyoplasts from the following sources were Used: 4- to 16-cell embryos

(embryonic nuclei), embryonic foetal, and adult (somatic) fibroblasts, embryonal tumour (Embryo Carcinoma) and embryonic stem cells (isolated from inner cell mass cells), embryonic disc cells, or primordial germ cells.

Karyoplast preparation from Preimplantation embryos.

Preimplantation embryos at 4 to 16-cell stage were collected from the reproductive tracts of superovulated and mated female miniswines. Superovulation was induced as described in Example 1. Foetuses for PGC isolation were collected from 25 to 35 day pregnant female miniswine following superovulation or natural cycling and mating (Example 1).

Four to 16-cell stage embryos were collected using standard surgical procedures 94 to 144 hours after hCG (Chorulon, Intervet (Aust) Pty. Ltd.) injection. Karyoplasts containing embryonic nuclei were obtained by removing a portion of the cytoplasm which contained the nucleus of the blastomere. This was performed by microsurgery in a similar manner to that described above for cytoplast preparation. The embryo was incubated in a microfilament inhibitor (e.g. cytochalasin B. (Sigma Cell Culture, Sigma-Aldrich Pty. Ltd.)) prior to the microsurgery, held in position by a holding pipette, the enucleation pipette was inserted between the blastomeres, and a portion of the cytoplasm containing the nucleus was removed. Karyoplasts were prepared from the blastomeres in the late G2 or M-phase of the cell cycle. The M- phase was induced by culturing embryos in a microtubule polymerisation inhibitor, for example nocodazole (Sigma Cell Culture, Sigma-Aldrich Pty. Ltd., 1 µg/ml) (Kwon and Kono, 1996). Alternatively the whole blastomeres, synchronised in the G2 or M- phase were used as the source of the karyoplasts. The cell cycle consists of four phases: M (mitosis), GO. S (synthesis) and G2. Synchronisation of cell cycles between donor nuclei and recipient cytoplasts at the time of transplantation strongly influenced the development of reconstituted eggs.

Karyoplast preparation from fibroblasts and embryonic cells.

Foetuses were collected on day 25 to 35 of pregnancy from slaughtered

female miniswine. The uterus was removed and foetuses isolated using sterile techniques. The foetuses were decapitated immediately after isolation and washed in cold PBS (Oxoid, Unipath Ltd. UK). A small tissue sample was taken from each foetus for genotyping (by using PCR). The fibroblast cultures were established from lung, muscle/skin (mesenchymal) tissues as follows. Other tissues may also be used. The tissue from individual foetuses was disected, washed in PBS (Oxoid, Unipath Ltd. UK), disaggregated mechanically by mincing with scissors or/and enzymatically by incubating for 5 min at 37°C in 0.25% trypsin / 0.04%/0 EDTA (Gibco BRL, Life Technologies inc. US) in PBS (Oxoid, Unipath Ltd. UK). Disaggregated cells were washed in culture medium and plated onto gelatinised 10 cm Petrie dishes, at 106 - 107 cells per dish. This is passage 0 (PO). The medium was changed every 24 hours and the cells cultured until confluent (between 2 to 5 days). The cells were then expanded by trypsinization and passaging 105 -108 cells onto a 10 cm gelatinised Petrie dish in culture medium. Aliquots of cells at passage 0, 1, 2 were stored frozen in liquid nitrogen. The culture medium was DMEM (Gibco BRL, Life Technologies Inc., US) supplemented with 10% FCS, 2 mM glutamine (Gibco BRL, Life Technologies inc. US), 100 IU/ml penicillin (Gibco BRL, Life Technologies Inc., US), 100 µg streptomycin (Gibco BRL, Life Technologies inc. US), 0.1 mM β mercaptoethanol (Gibco BRL, Life Technologies inc. US). Other types of media or protein/growth factors may also be used (e.g. DMEM (Gibco BRL, Life Technologies Inc., US):F12 (Gibco BRL, Life Technologies Inc. US)) if they are found to provide better culture conditions. The cells were karyotyped at each passage to confirm that they retained normal/diploid chromosome complement.

Other types of cells used as a karyoplast source include tissue from adults and embryonic stem cells isolated from ICM, ED, PGC and EC.

PBS

Sterile solution of PBS (Oxoid, Unipath Ltd. UK), (Ca^{++} and Mg^{++} free) supplemented with 1 00 IU/ml penicillin, 100 µg/ml streptomycin.

Culture media

Dulbecco's modified Eagle's medium (DMEM, (Gibco BRL, Life Technologies Inc. US)) supplemented with 10-20% FCS (CSL Limited, Aus), 100 IU/ml penicillin (Gibco BRL, Life Technologies Inc. US), 100 µg/ml streptomycin (Gibco BRL, Life Technologies Inc. US), 2 mM glutamine (Gibco BRL, Life Technologies Inc. US), 0.1 mM mercaptoethanol (Gibco BRL, Life Technologies Inc. US).

Gelatin

0.1% gelatin (Sigma Cell Culture, Sigma-Aldrich Pty. Ltd.) in water.

Trypsin

0.25% trypsin (Gibco BRL, Life Technologies Inc. US), 0.04% EDTA (Gibco BRL, Life Technologies Inc. US) in PBS (Oxoid, Unipath Ltd. UK), (without Pen/Strep).

Freezing

In 90% culture medium, 10% DMSO (Sigma Cell Culture, Sigma-Aldrich Pty. Ltd.).

EXAMPLE 3

Isolation and genetic modification of bovine embryonic fibroblasts

Isolation and propagation

A bovine foetus (CR length 7.5 cm) was collected from an abattoir - slaughtered cow. The foetus was washed in 3 changes of cold PBS containing 100 IU/ml penicillin, 100 µg streptomycin (PBS-P/S) eviscerated and washed again in 3 changes of cold PBS-P/S. Fibroblast cultures were established from

superficial skin (BEF-S) and from muscle (mesenchymal; BEF-M) tissues as follows. The superficial skin was peeled off from hind and front legs and the muscle tissue was disected from hind legs, washed in 2 changes of cold PBS-P/S, disaggregated mechanically by chopping with scalpel blades followed by enzymatic digestion. For enzymatic digestion tissues were incubated for 90 min (or up to 4 - 6 hr) at 4°C in 0.25% trypsin / 0.04% EDTA in PBS. After removing the excess of trypsin, tissues were incubated at 37°C for 10 - 20 min, culture medium was added and cells dissagregated by vigorous pipetting. Large, undigested pieces of tissue were allowed to settle by sedimentation. Supernatant containing single cells was decanted and the cells plated at 3×10^4 cells/cm^2 onto gelatinised (0.1% gelatin in water) Petrie dishes and cultured at 37°C in an atmosphere of 5% CO_2 in air. This was passage 0 (P0). The medium was changed every 24 hours and the cells cultured until confluent (between 2 to 5 days). The cells were then expanded by trypsinization and passaging 10^5 - 10^6 cells onto a 10 cm gelatinised Petrie dish in culture medium. Aliquots of cells at passage 0, 1, 2 were stored frozen in liquid nitrogen (in 10% DMSO, 90% culture medium or FCS). The culture medium was DMEM supplemented with 15% FCS, 2 mM glutamine, 100 IU/ml penicillin, 100 µg streptomycin, 0.1 mM β mercaptoethanol.

PBS : sterile solution of PBS (Ca^{++} and Mg^{++} free) supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin

culture medium : Dulbecco's modified Eagle's medium supplemented with 15% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 0.1 mM β mercaptoethanol

gelatin : 0.1% gelatin in water

Trypsin : 0.25% trypsin, 0.04% EDTA n PBS (without Pen/Strep)

Freezing medium : 10% DMSO, 90% culture medium or FCS

Sex determination

The sex of the isolated fibroblasts was determined by PCR (Pomp et al J. Anim Sci 1995; 73: 1408-1415). Two genes were amplified in a single reaction:

Sry (sex-determining region Y); the presence or absence of this gene determines sex;

Zfy (male) or Zfx (female); the amplification of these genes acts as a positive control for PCR.

The cells were found to be isolated from male foetus.

Genetic modification

Muscle tissue derived fibroblasts were used to introduce gene markers: β-galactose gene and/or neomycin gene. The cell line, BEF-M, was transfected by electroporation with the following vectors:

a) 6PLacZ TINβS-MO linearized at a unique Xho I restriction site (β-galactosidase-neomycin resistant fusion gene), or

b) IRES-Neo linearized at a unique Mlu I restriction zite (neomycin resistance gene).

2×10^6 BEF-M cells were suspended in 0.4 ml of HeBs (20 mM HEPES-NaOH pH 7.05, 137 mM NaCl, 5 mM Kcl, 0.7 mM Na_2HPO_4, 6 mM glucose) containing 10 µg of linearized vector. The cell suspension was placed in a 4 mm gap electroporation cuvette and kept at room temperature for 10 min. A 200 V, 950 µF charge was then applied with a Gene Pulser II apparatus (Bio-Rad Laboratories). After 10 min incubation at room temperature the cell suspension was transferred to 10 ml of culture medium and plated onto 10 cm Petri dishes. The cells were re-fed culture medium containing 400 µg/ml Geneticin (Life

Technologies) and selected for 8 to 14 days.

Following electroporation and selection with Geneticin, 18 6PLacZ TINβS-MO and 21 IRES-Neo stable transfectants were isolated. The positive clones were expanded and stored frozen in liquid nitrogen.

The positive 6PLacZ TINβS-MO clones were analysed for the expression pattern of β-galactosidase gene by staining with X-gal. One clone, where ≥ 90% of cells strongly expressed the marker gene, was chosen for nuclear transfer experiments.

EXAMPLE 4

Isolation of porcine adult fibroblasts

Isolation and propagation

Ear samples were collected from adult male and female miniature swine. The tissue samples were briefly sterilised in 70% ethanol and washed in 3 changes of cold PBS containing 100 IU/ml penicillin, 100 µg streptomycin (PBS-P/S). The fibroblast cultures were established from skin as follows: The skin was disaggregated mechanically by chopping with scalpel blades followed by enzymatic digestion. For enzymatic digestion tissues were incubated for 5 hr (or up to 12 hr) at 4°C in 0.25% trypsin / 0.04% EDTA in PBS. After removing the excess of trypsin, tissues were incubated at 37°C for 30 min, culture medium was added and cells disaggregated by vigorous pipeting. Large, undigested pieces of tissue were allowed to settle by sedimentation. Supernatant containing single cells was decanted and the cells plated onto gelatinised (0.1% gelatin in water) Petrie dishes and cultured at 37°C in an atmosphere of 5% CO_2 in air. This was passage 0 (P0). The medium was changed every 24 hours and the cells cultured until confluent (between 5 to 8 days). The cells were then expanded by trypsinization and passaging 10^5 to 10^6 cells onto a 10 cm gelatinised Petrie dish in culture medium. Aliquots of cells at passage 0, 1, 2 were stored frozen in liquid

nitrogen (in 10% DMSO, 90% culture medium). The culture medium was DMEM supplemented with 15% FCS, 2 mM glutamine, 100 IU/ml penicillin, 100 µg streptomycin, 0.1 mM β mercaptoethanol

PBS : sterile solution of PBS (Ca^{++} and Mg^{++} free) supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin

culture medium : Dulbecco's modified Eagle's medium supplemented with 15% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 0.1 mM β mercaptoethanol

gelatin : 0.1% gelatin in water

Trypsin : 0.25% trypsin, 0.04% EDTA n PBS (without Pen/Strep)

Freezing medium : 10% DMSO, 90% culture medium or FCS

EXAMPLE 5

Gene constructs and transgenesis

The genetic modification of animal genomes can be broadly divided into random and targeted transgene integration. While random transgene integration suffers considerable limitations attributable to unpredictable site of integration effects, targeted transgene integration offers significant improvement in current loss of function applications and significantly greater opportunity in enabling planned modification of specific endogenous genes.

To date, the generation of transgenic animals with targeted transgene integrations has been restricted to mice. Targeted mutations in mice have been possible due to the availability of ES cell culture systems which are not available for other species. ES cells, which are isolated from the inner cell mass of preimplantation mouse embryos, can be extensively propagated *in vitro* without losing their capacity to contribute to all cell types of an animal. The *in vitro*

propagation of ES cells provides an extremely valuable opportunity to introduce transgenes and select clonal cell lines having the desired transgene integration event. The selected cell line can then be used as a genetic resource to establish an equivalent transgenic animal.

An alternate route to ES cell-mediated transgenesis is to couple *in vitro* cell culture and genetic manipulation with nuclear transfer. Nuclear transfer differs from ES cell-mediated transgenesis in that a variety of cell types that can be cultured and genetically manipulated *in vitro* can be utilised as a genetic resource for the establishment of a new animal. For example, embryonic fibroblasts which can be propagated and genetically manipulated *in vitro* are not pluripotential and cannot contribute to the germline of an animal. However, the nuclei of genetically modified embryonic fibroblasts can be utilised as a genetic resource to establish a transgenic animal by reprogramming the developmental capability of that nucleus through nuclear transfer. Conversely, fertilised oocytes or single cells of *in vitro* or *in vivo* produced embryos can be cultured, genetically modified and used as a genetic resource for generating transgenic animals by nuclear transfer or aggregation with host embryos.

The introduction of targeted genetic modifications in specific genetic loci is facilitated by a process known as homologous recombination. Specific genetic changes can be introduced into a locus by flanking the new sequence with genomic sequences homologous to the target site. The homologous sequences recombine with the target endogenous gene and introduce the engineered mutation. To introduce specific targeted mutations in the germline of inbred MHC (SLA) miniature swine, we have engineered MHC (SLA) miniature swine genomic libraries and cloned and engineered a range of gene targeting vectors as follows. Figure 2 summarises the steps taken for construction of the pGallaway targeting vector.

Endogenous genes are not limited to the examples shown. Gene targeting and nuclear transfer in swine and other animals would also be useful in cases of:-

1) Removal (knockout) of genes responsible for boar taint,
2) Disruption of the myostatin gene to increase muscle growth,

3) Growth modification: directing modified growth hormone construct into the growth hormone locus,

4) Using gene expression on the X and Y chromosomes to alter the ratio of males to females.

5) Addition of transgenes to increase disease resistance, interferon gene,

6) Production of human pharmaceuticals,

7) Disease free transfer of genetic blood lines,

8) Derivation of disease free swine from any genetic blood line,

9) Derivation of specific pathogen free (SPF) swine genetic blood lines.

MHC (SLA) Miniature swine genomic DNA library construction

Genomic DNA from a d/d miniswine shown to be homozygous at the α1,3-galactosyltransferase locus by RFLP analysis was used to construct a genomic library. The same swine was used for derivation of the target cells used in these studies, assuring isogenicity and target locus. The lambda genomic library was made from DNA isolated from liver tissue using the Promega LambdaGEM-12 Xho I Half Site Arms (Promega Corp., Madison, WI) protocol with modifications. The genomic DNA was partially digested with Sau 3A I followed by partially filling-in of the Sau 3A I as described by Promega. The DNA fragments were then size fractionated by agarose gel electrophoresis using standard methodologies. The DNA was ligated to LambdaGEM-12 vector, which had already been digested with Xho I and dephosphorylated; and the first two nucleotides of the Xho I site filled in. The ligated DNA was packaged using the Stratagene Gigapack Gold Packaging Extract and mixed with E.coli host strain, KW251. The genomic library contained approximately 2×10^6 independent clones.

Construction of vector, pBert

The unamplified library was screened with a 240 bp PCR fragment corresponding to nucleotides 607 through 847 of porcine α1,3-galactosyltransferase gene (GT. Strahan et al. 1995; immunogenetics, vol. 41: 101-105, cDNA Sequence and Chromosome Localization of swine

α1,3-galactosyltransferase, Genbank Accession Number L36152) using standard methodologies. Six bona fide porcine α1,3-galactosyltransferase clones were identified and mapped by Southern analyses using standard methodologies. Two clones (#12 and 4) were selected for use in construction of the targeting vector, pGallaway.

The two lambda clones were subcloned as follows: lambda clone 12 was digested with Eco RI and the 10 kb fragment containing GT sequences upstream of exon 9 was isolated. This fragment was cloned into pUC19 Eco RI cut vector to generate pTCL12-45 (orientation 1) and pTCL12-40 (orientation 2). Lambda clone 4 was digested with Sac I and the 11.8 kb fragment containing GT sequences downstream of exon 9 was isolated, then cloned into pNEB 193 (NEB) Sac I cut vector to generate pTCR4-9. A 2.8 kb Eco RI/Sac I fragment was also isolated from lambda clone 12 and cloned into pNEB 193 Eco RI/Sac I cut vector to generate 4-2. This fragment contains 3' intron 8 sequences and 5' exon 9 sequences. Together, the 10 kb Eco RI fragment (clone 12), the 2.8 kb Eco RI/Sac I (clone 12) and the 11.8 kb Sac I fragment (clone 4) form a 24.6 kb contiguous fragment containing the porcine GT extending from intron 7 through exon 9 into sequences 3' of GT.

In order to introduce stop codons into the 5' end of exon 9, pBERT (Figure 3) was made. The plasmid, pTCL12-45, was digested with Eco RI and Sac I, the 7.5 kb fragment containing the GT region was isolated, a linker (comprised of oligonucleotides 5'-TCG ACT GTT TAA ACG GCC TCT ACG GCC TAG CT-3' (SEQ ID NO:1) and 5'-AGG CCG TAG AGG CCG TTT AAA CAG-3' (SEQ ID NO:2)) as shown in Figure 4 with cohesive Sal I and Sac I ends, which contained a unique Pme I and Sfi I was ligated to the fragment, followed by ligation to pUC19 Eco RI/Sal I digested vector to generate pTCL12A. This vector lacks about 2.5 kb of the 5' end of the original lambda 12 clone. Stop codons were introduced into the 5' end of exon 9 by modifying 4-2. The plasmid, 4-2, was digested with Bse RI to drop out a 94 bp internal GT Bse RI fragment. This fragment was replaced with a synthetic fragment which was modified such that maximum isogenicity was maintained. Five bp changes were introduced into this synthetic Bse RI fragment.

Two bp modifications changed the 5' Bse RI (within intron 8) to a unique Sal I site, while 3 bp changes created three in-frame stops within the first 50 bp of exon 9 (See Figure 5). The synthetic Bse RI fragment had cohesive Bse RI ends and was ligated to 4-2 Bse RI cut vector to generate 4-2A.

The vector, 4-2A, was digested with Eco RI and Sac I and the 2.8 kb Eco RI/Sac I fragment containing the modified Bse RI region was isolated. Vector, pTCL12A, was digested with Eco RI, dephosphorylated, and ligated together with the 4-2A Eco RI/Sac I fragment. The ligatin reaction was first digested with Pme I, then with Sac I, and the 9-10 kb Pme I/Sac I fragment was purified. The vector, pTCR4-9, was digested with Not I, dephosphorylated, digested with Sac I and the resulting 11 kb Sac I/Not I fragment was purified. The vector, (SuperCos) Stratagene), was digested with Eco RI, which was filled-in with Klenow, then digested with Xba I and the 6.5 kb SuperCos ori fragment isolated. A second vector, SuperCos:stuffer, which contains the 11.4 kb Cla I fragment from EMBL3 cloned into the Cla I site of SuperCos, was digested with Xba I and Not I and the 12.6 kb Xba I/Not I fragment isolated.

The four purified fragments: 6.5 kb SuperCos ori (Eco RI)/Xba I; 12.6 kb SuperCos:stuffer Xba I/Not I; 9-10 kb TCL12A/4-2A Pme I/Sac I; and 11 kb TCR4-9 Sac I/Not I were ligated together in a two-step ligation. The TCL12A/42A fragment was first ligated to the TCR4-9 fragment and then the remaining two fragments were added to the ligation. The ligation mixture was packaged as described earlier. Clones were screened using standard methodologies and one correct clone, pBERT was used for further work.

Construction of pGallaway

In order to clone to 5' region of GT, pTCL12-40 was digested with Eco RI, which was filled-in with Klenow, followed by digestion with Xho I. The resulting 6.5 kb fragment containing the 5' GT region was purified. The recipient vector (pOCUS/pgk-neo) is a modified pOCUS vector (Novagen) which contains a pgk-neo cassette cloned into the Hinc II site of pOCUS. A partial Spe I digest was carried out on pOCUS/pgk-neo, the linearized Spe I vector purified, then digested

with Xho I. The resulting 3.7 kb fragment containing the vector was purified and ligated to the 6.5 kb (Eco RI)/Xho I fragment from pTCL12-40 to generate pEddie.

The 3' GT region was cloned into pEddie as follows: pEddie was digested with Xho I and Not I, dephosphorylated and the 10 kb fragment purified. pBert was digested with Xho I and Not I which removed the 18.1 kb GT region (with stop codon modifications) from the vector backbone. The unpurified 18.1 kb Eco RI/Not I fragment was ligated to the pOCUS/pgk-neo Eco RI/Not I vector. The ligated DNA was electroporated into competent E.coli TOP10 cells and individual colonies screened by standard methodologies. One correct pGallaway clone was identified and was used for further work.

pGallaway can be used as both a replacement or insertion vector. Either the unique Xho I or Sal I sites within the GT region can be used to linearize pGallaway for use as an insertion vector. The entire 24.6 kb GT region can be removed from vector sequences by digestion with Sfi I and Not I.

Several criteria were applied to the selection of an appropriate cell line in which to assess homologous recombination with the pGallaway vector. First, the line must be isogenic with the vector. Second, the line can be immortalised to permit sufficient passage between the initial transfection and subsequent analyses. Third, the line should express the α1,3-galactosyltransferase gene, as expression will be required for the homologous recombination assay. Fourth, the line must be transfectable and selectable at reasonable frequencies. Fifth, to facilitate generation of an α1,3-galactosyltransferase null line the line should be diploid.

Aortic endothelial cells from the same miniature swine used to derive the genomic DNA library were immortalised with SV40 T antigen (Seebach et al., 1997, The 4th International Congress for Xenotransplantation, Nantes, France). One of the cell lines, PEDSV15, which satisfied the criteria listed above, was transfected by electroporation with the pGallaway targeting vector. The vector was linearized at a unique Xho I restriction site. 1 x 10^7 PEDSV15 cells were suspended in 0.8 ml of HeBS (20 mM HEPES-NaOH pH 7.05, 137 mM NaCl,

5 mM KC1, 0.7 mM Na_2HPO_4, 6 mM dextrose) containing 0.5 nM linearized pGallaway. The cell suspension was placed in a 4 mm gap electroporation cuvette and chilled on ice. A 270 v, 980 uF charge was then applied with a Gene Pulser apparatus (Bio-Rad Laboratories, Hercules, CA) and the cuvette returned to ice for 10 minutes. The cell suspension was then transferred to 10 ml of standard medium (M199 containing 10% fetal bovine serum (Life Technologies, Gaithersburg, MD) and pelleted. The cell pellet was resuspended and plated in enhanced medium (M199, 20% FBS, 100 µg/ml Endothelial Cell Growth Supplement (Collaborative BioMedical, Bedford, MA)) at approximately 6 x 10^4 cell/cm^2 in various size plates to generate pools with varying numbers of stable transfectant clones, a limiting dilution series was performed to determine the stable transfection frequency for extrapolation of stable clones/plate. The cells were re-fed enhanced medium containing 400 µg/ml Geneticin (Life Technologies, Gaithersburg, MD) and selected for 10-14 days.

RT-PCR Assay for Homologous Recombination

The genomic organisation of the α1,3-galactosyltransferase gene following a homologous recombination event between the chromosomal locus and the pGallaway vector is shown in Figure 6. The length of the α1,3-galactosyltransferase homologous sequences in pGallaway (approximately 12 Kb on either side of the changes introduced in exon 9) precluded use of a simple DNA PCR assay for identifying cells which had undergone a homologous recombination event. Additionally, to avoid the necessity of generating clonal lines of stable transfectants for assay, the assay had to specifically identify homologous recombination events within pools of stable transfectants also containing non-homologous insertions of the targeting vector.

Homologous recombination of the pGallaway targeting vector in endothelial cells should result in the generation of RNA transcripts containing the mutations (stop codons) introduced into the vector. These transcripts are most readily identified by RT-PCR using a forward primer from an α1,3-galactosyltransferase exon upstream of the vector sequences and a reverse primer specific for

amplification of the expected mutant transcript. The forward primer chosen for these experiments was derived from wild-type exon 7 sequence. The reverse primer was derived from the mutated region of exon 9 in the pGallaway vector, and has a 3' terminal mismatch to the wild-type α1,3-galactosyltransferase sequence. RNA was prepared from stable transfectant cells using the RNeasy Isolation Kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. cDNA was prepared from RNA using the Superscript Preamplification System (Life Technologies, Gaithersburg, MD), using random hexamers according to the manufacturer's instructions. cDNA was amplified in 100 Pl reactions containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 10 µM each dNTP, 0.9 mM $MgCl_2$, and 25 µ/ml Amplitaq Gold polymerase (Perkin-Elmer, Norwalk, CT). Forward primer F238 5'-TTA CCA CGA AGA AGA AGA CGC-3' (SEQ ID NO:3) and reverse primer RD2 5'-TGC AGA TAT TCA GAA CTC CTC CT-3' (SEQ ID NO:4) were present at 200 nM. Amplification reactions were performed using a System 9600 thermocycler (Perkin-Elmer, Norwalk, CT) with the following profile:

1) 95°C for 9 min.
2) 39 cycles of:
   96°C for 2 sec
   60°C for 30 sec
   72°C for 30 sec
3) 72°C for 5 min.

Analysis of Targeting Efficiency

Two weeks following electroporation and selection with Geneticin aliquots of the stable transfectant pools were analysed by RT-PCR as follows:

To verify that amplifiable cDNA was prepared from the pools, the cDNA reaction was amplified with wild type primers F238 and R545 5'-AGA TGA CTT TGT GGC CAA CC-3' (SEQ ID NO:5), from porcine α1,3-galactosyltransferase exons 7 and 9 respectively.

To ensure that the RNA pools were not contaminated with mutant template, the mock cDNA reactions were amplified with primers F238 and RD2.

To identify pools containing cells producing the mutant transcript expected from the homologous recombination of pGallaway and the endogenous α1,3-galactosyltransferase locus, the cDNA reactions were amplified with primers F238 and RD2.

Initial analysis of 7 pools of approximately 500 stable transfectants each resulted in detection of the expected mutant template in all 7 pools. A subsequent analysis was performed on 6 pools of 30 stable transfectants (21 A-F) and 4 pools of 3 stable transfectants (22 A1,B1,C1,D1). The results of this analysis are shown in Figure 8. In panel A, ethidium bromide staining of PCR products generated from cDNA with the wild type F238 and R545 primers showed that amplifiable cDNA was produced from each pool.

In panel B, the pool cDNA preparations were amplified with primers F238 and RD2, for specific detection of product generated from the expected mutant transcript. Hybridization of Southern blots with an internal α1,3-galactosyltransferase oligonucleotide probe revealed the presence of mutant transcripts in 2/6 pools of 30 (21 B,C). In panel C, mock cDNA amplified with primers F238 and RD2 confirmed the absence of contamination within any of the pools analysed.

To eliminate any possibility that the PCR product generated from pools 21 B and 21 C was derived by low efficiency amplification of wild type RNA, the PCR product from these pools was digested with Ase I. Ase I should cleave the expected mutant product into fragments of 272 bp and 24 bp, but not cleave product derived from a wild type template (see Figure 7). The polyacrylamide gel analysis in Figure 9 clearly demonstrates that the PCR product from pools 21 B and C is cleaved, confirming the amplification of the mutant transcript expected from a homologous recombination event.

In summary, 2/6 pools of 30 stable transfectant clones were found to have mutant transcripts expected from a homologous recombination event, for a homologous recombination frequency of 1-2%.

To select for a cell line totally devoid of α1,3-galactosyltransferase activity, ie., a null cell line, we reasoned that screening the pools of clones for lack of binding to antibodies specific for the Galα1,3Gal epitope, synthesised by α1,3-galactosyltransferase might result in selection of such a null cell line. Accordingly, we subjected pools of the stable transformant cell lines to multiple rounds of selection using baboon xenogenic natural antibodies (XNAs) eluted from a Galα1,3Gal column matrix (Synsorb 90, Alberta Research Council. Canada) and rabbit complement. Following five rounds of selection, the vast majority of cells in one pool (2*3) were resistant to lysis. RT-PCR analysis was performed on RNA from the 2*3 pool prior to selection and after five rounds of selection. As expected from the size of the pools, only wild type PCR product was detected in the 2*3 pool prior to selection. Following selection, the vast majority of PCR product was that expected from the targeted allele. Small amounts of PCR product derived from the wild type RNA are compatible either with a small surviving sub-population of GT expressing cells or with a significant, but not total, loss of wild type RNA in the "null population". A FACS analysis was performed on the 2*3 line following 7 rounds of XNA/complement selection, and the parental PEDSV15 line to determine the relative levels of binding to Synsorb 90 or to the lectinIB4, which has binding specificity to Galα1,3Gal epitope. In both cases, the 2*3 cell line showed a greatly reduced level of binding, indicating that the cell line had a greatly reduced level of expression of the α1, 3-galactosyltransferase activity.

Having demonstrated that gene targeting of the α1,3-galactosyltransferase locus can be achieved in a porcine sortie endothelial cell line, it is understood that other cell types can be similarly modified, including but not limited to cultured cells, eg., embryonic fibroblasts, or embryonic stem cells, or embryos.

The introduction of new genetic material and the subsequent selection of cells harbouring the desired targeted integration requires expansion and clonal selection of each founder transgenic cell. A limitation to applying this processes in nuclear transplantation programs is the number of cell divisions which the transfected cell must undergo to provide sufficient material for molecular analysis of each transgenic colony and subsequent supply of nuclei for transfer. The great

majority of cells suitable for *in vitro* genetic modification and subsequent nuclear transfer have limited *in vitro* propagation capacity. It is therefore desirable to utilise transfection and selection systems which generate and/or identify correctly targeted clones at high efficiency and with limited requirement for *in vitro* propagation.

A particularly efficient approach to selecting for correctly targeted clones is to use IRES gene trap targeting vectors. IRES gene trap targeting vectors provide a significant enhancement in gene targeting efficiency by eliminating a large proportion of random integration events. IRES gene trap targeting vectors rely upon functional integration into an actively transcribed gene (such as the target gene) for expression of the selectable marker. Random integrations into non-transcribed regions of the genome are not selected.

Widely expressed genes, such as the α1,3-galactosyltransferase gene which is the principal target for gene deletion in xenotransplantation, is well suited to IRES gene trap targeting strategies in nuclear transfer programs. Figure 10 shows one α1,3-galactosyltransferase IRES gene trap targeting vector for functional disruption of α1,3-galactosyltransferase gene expression. In a preferred embodiment, it may be desirable to remove the selectable marker cassette from the targeted locus to eliminate expression of the antibiotic resistance gene. One approach is to flank the IRES selectable marker cassette with suitable DNA sequences which act as recombination sites following the addition of a suitable site-specific recombinase. One example of a suitable recombinase site is the lox site which is specific for the Cre recombinase protein. Figure 11 shows one example of a recombinase sensitive α1,3-galactosyltransferase IRES gene trap targeting vector for functional disruption of α1,3-galactosyltransferase gene expression.

High efficiency gene targeting and selection has a significant advantage in that suitably stringent selection systems, such as the IRES gene trap targeting vectors, can eliminate the need for biochemical analysis of clonal cell lines. In this instance, individual nuclei from a pool of uncharacterised transgenic cells should

generate offspring of the desired phenotype at a ratio equivalent to the selected pool. The elimination of clonal selection may be particularly useful where only limited *in vitro* propagation is desirable or possible. One such instance includes the culture of embryonic nuclei for nuclear transfer. Embryonic nuclei are more efficient than latter stage somatic cells for generating live born offspring by nuclear transfer, however, totipotential embryonic cells can not be cultured for extended periods for any other species than mice. Nuclear recycling of embryonic nuclei (see following) provides an opportunity to maintain, expand and genetically manipulate multipotential cells from swine *in vitro* in the absence of typical ES culture systems.

Transgenes may be introduced into cultured cells or embryos by direct injection, electroporation, liposomes or calcium phosphate precipitation. Direct injection is the preferred method for individual embryonic cells while electroporation is more suitable for embryonic fibroblasts and embryonic stem cells. Typically, plasmid DNA for electroporation was linearised by restriction enzyme digest, ethanol precipitated and resuspended at 1.0 mg/ml in PBS (Oxoid, Unipath Ltd. UK). Following 2 hours culture in fresh medium, near confluent cells were dispersed by trypsinisation, washed sequentially in culture medium and PBS (Oxoid, Unipath Ltd. UK), and resuspended at 1.4x10^8/ml in PBS (Oxoid, Unipath Ltd. UK) for immediate transfection. Routinely, 0.7 ml of cell suspension was mixed with 0.1 ml DNA containing solution and electroporated at 0.8 kV and 3.0 mFD using a Biorad Gene Pulser and 0.4 cm cuvettes. Transfections were plated on gelatinised tissue culture dishes at 5-8x10^4/cm2 in growth medium for 16 hours prior to the addition of selection medium containing 200 mg/ml (active) G418 (Sigma). Single colonies were picked 8-10 days post transfection and transferred in duplicate into 24 well tissue culture plates for further expansion in growth medium containing 200 mg/ml G418 and subsequently in media without G418.

EXAMPLE 6

Elimination of α1,3-galactosyltransferase activity in porcine foetal

fibroblasts

Mesenchymal tissue derived fibroblasts were used to eliminate α1,3-galactosyltransferase activity. The cell line, PEM, was transfected by electroporation with the pGallaway targeting vector linearized at a unique Xho I restriction site. 1 - 4.8 x 10^6 PEM cells were suspended in 0.4 ml of HeBs (20 mM HEPES- NaOH pH 7.05, 137 mM NaCl, 5 mM Kcl, 0.7 mM Na_2HPO_4, 6 mM glucose) containing 0.5 mM linearized pGallaway. The cell suspension was placed in a 4 mm gap electroporation cuvette and kept at room temperature for 10 min. A 200 V, 960 µF charge was then applied with a Gene Pulser II apparatus (Bio-Rad Laboratories). After 10 min incubation at room temperature the cell suspension was transferred to 10 ml of culture medium and plated onto a number of 10 cm Petri dishes. The cells were re-fed culture medium containing 400 µg/ml Geneticin (Life Technologies) and selected for 8 to 14 days.

Following electroporation and selection with Geneticin aliquots of the stable transfectants were analysed by RT-PCR as described in Example 3. Two sets of primers were used:

- wild type (primers F238 and R545) to verify that cDNA was transcribed successfully;
- mutant (primers F338 and RD2) to identify the cells producing the mutant transcript expected from the homologous recombination of pGallaway and the endogenous α1,3-galactosyltransferase locus.

In two electroporation experiments 7.6 x 10^6 were transfected and 178 Geneticin resistant colonies isolated and analysed for the elimination of one copy of the α1,3-galactosyltransferase gene. Five individual targeted PEM clones have been identified, expanded and stored in liquid nitrogen. Four of these clones have been used for nuclear transfer so far.

The following strategy has been developed to identify and isolate primary clones harbouring targeted integrations. To prevent extensive culture of targeted

colonies a "master" plate is stored frozen, while duplicate plate(s) are expanded and progressively smaller pools of colonies analysed by RT-PCR for the targeted event.

The "master" plate is frozen as follows: When the cells reach confluency, the medium is removed and cells briefly washed with PBS, and 0.25% trypsin, 0.4% EDTA in PBS. The plate is then put on ice and 500 µl of cold freezing medium (10% DMSM, 90% FCS) added to each well. The plate is stored for up to 2 to 4 weeks at -75°C or after 24 hr at -75°C transferred to liquid nitrogen. To thaw the cells, the plate is transferred to room temperature, 500 µl of warm culture medium added to each well and the cell suspension transferred to a new 24 well plate. The cells are cultured at 37°C in an atmosphere of 5% CO_2 in air.

EXAMPLE 7

Nuclear Transfer

Activation

Activation of the cytoplast was preferred for further embryonic development. Activation occurs during fertilisation and is characterised by a series of Ca^{2+} oscillations, release of cortical granules, extrusion of a second polar body, pronuclear formation and ultimately cleavage. Development of an oocyte without fertilisation by sperm is known as parthenogenic activation. Parthenogenic activation can be induced by various chemical and physical treatments such as ethanol, calcium ionophore and electrical pulses (Ozil, J.P. 1990). In the swine, electrical pulses have been shown to be more effective at induction of parthenogenetic development than ethanol (Saito et al., 1993). Electrical stimulation is therefore the preferred method of activation induction. Oocytes and reconstituted cytoplasts were activated by exposure to one or a series of electrical pulses (DC) at 90-120 V/mm for 30-90 µs (Prather et al 1991).

The activated reconstituted oocytes were cultured *in vitro* overnight (24

hrs), or to the blastocyst stage (Day 6) of development (Example 6) prior to transfer into surrogate swine (Example 8)

TABLE 3

Parthogenetic activation of porcine *In Vitro* and *In vivo* matured oocytes using different methods

Treatments	No. of Oocytes Treated	No. of Oocytes cleaved to 2-4 cell stage. (%)	No. of Oocytes cleaved to Morula/Blastocyst. (%)
Ethanol (10%, 15 minutes)	118	53 (44.9)	
Calcium Ionophore (CI)	100	48 (48.0)	
Cycloheximide (10µg, 20 hours)	75	23 (30.7)	
Calcium Ionophore + Cycloheximide	59	29 (49.2)	
DC pulse	248	137 (55)	In Vitro 8/65 (12%) In Vivo 7/59 (11%)
Control	60	9 (15)	

Fusion

The nuclear transfer procedure involved the reconstitution of an enucleated cytoplast with a karyoplast containing the donor nucleus. Cytoplasts were reconstituted by inducing membrane fusion with the karyoplast following treatment with chemical reagents, such as polyethyleneglycol (Czolowska et al., 1984), or by exposure to electrical pulses (Willadsen 1986). Cytoplasts were also reconstituted by direct injection of the intact donor nucleus and karyoplast lysate into the

cytoplast (Bromhall 1975; Illmensee and Hoppe 1981).

Electrofusion was the preferred technique for cytoplast reconstitution. The parameters required for successful fusion depends on the origin of the karyoplast and also whether the zonae pellucida was present on the cytoplast. When cytoplasts with an intact zonae pellucida were used, the karyoplast was inserted into the perivitelline space (PV) of the cytoplast between the oocyte membrane and zone pellucida, with all manipulation performed in cytochalasin B (Sigma Cell Culture, Sigma-Aldrich Pty. Ltd., 5 µg/ml). To induce fusion, the cytoplast and karyoplast were placed between two platinum electrodes 0.5 - 1.0 mm apart in a solution of 0.3 M mannitol (Sigma Cell Culture, Sigma-Aldrich Pty. Ltd.), (Prather et al., 1989). For effective fusion to occur, the cytoplast and karyoplast were orientated in a perpendicular fashion so that the fusion plane of the two cells was parallel to the electrodes. An alignment current of 5-10 V AC (500-800 kHz for less than 10 s) was used to assist in orientation. When aligned, fusion was induced by a brief exposure to DC pulse (30-120 V/mm for 50 - 300 µs). Fusion of the cytoplast and karyoplast was observed within 15 -30 minutes by loss of membrane distinction between cells.

Cytoplast volume has been demonstrated to effect development of nuclear transfer embryos (Peura et al 1997). It may therefore be beneficial to increase the cytoplast volume at fusion, especially when cytoplasts have been prepared by oocyte bisection (see Example 1). To increase cytoplast volume, zona pellucida free cytoplasts may be fused together before, after, or at the same time as karyoplast fusion using fusion parameters and conditions described above. Alternatively, optimal fusion parameters can be determined by the establishment and evaluation of isofusion contours.

A comparison of our nuclear transfer procedures in the pig is shown in Table 4. Many attempts at using existing published nuclear transfer approaches developed in sheep. (Campbell et al., 1997) and cattle (Cibelli et al., 1998) were unsuccessful for the pig.

The techniques that resulted in normal embryo development in a

reconstituted "nuclear transfer" porcine embryo include

1) porcine oocytes are activated prior to enucleation, followed immediately by insertion of the donor nuclei and fusion 3 - 6 hrs after activation, and

2) modification of technique 1) where oocyte are enucleated (without activation), followed immediately by insertion of the donor nuclei with combined fusion activation occurring 3 - 6 hours after enucleation.

TABLE 4

Efficiency of Nuclear Transfer in Pigs

Procedure	Enucleation (%)	Fusion (%)	Development (%)	
			2/4-cell	Morula/Blastocyst
Activation - Enucleation - (6 hrs) Fusion	166/268 (62)	136/166 (82)	90 (66)	In Vitro 15/67 (22) In Vivo 5/42 (12)
Enucleation – (6 hours) – Activation/ Fusion Combined	72/89 (81)	49/72 (68)	19/42 (38)	In Vitro 3/19 (16)
Published methods for sheep and cattle	Unsuccessful			

Table 5 shows a summary of embryo transfer results for surgical transfer of reconstituted nuclear transfer porcine embryos produced by techniques of the present invention to synchronized recipients.

TABLE 5

Transfer of porcine NT embryos

Pig ID	Embryo Transfer	Recipient Type	Location	Cytoplast Source	No. of NT embryos	NT cell line (minipig)	Return to Oestrus	Ultra-sound (35 days)	Status	Date of Return	Preg. Detect. (Days)
RT 223	8/7/98	Outbred	VIAS	*in vivo*	29	1G5 P4 (WT)	28/7/98	11/8/98	Returned	28/7/98	
RT 224	9/7/98	Outbred	VIAS	*in vivo*	19	1G5 P4 (WT)	29/7/98	12/8/98	Returned	28/7/98	
RT 225	10/7/98	Outbred	VIAS	*in vivo*	13	1G5 P4 (WT)	30/7/98	13/8/98	Returned	12/10/98	94
RT 226	30/7/98	Outbred	VIAS	*in vitro*	20	1G5 P4 (WT)	19/8/98	2/9/98	Returned	13/10/98	75
RT 227	30/7/98	Outbred	VIAS	*in vitro*	20	1G5 P4 (WT)	19/8/98	2/9/98	Returned	9/9/98	41
RT 228	31/7/98	Outbred	VIAS	*in vitro*	15	2M 15/5; 1-9(KO)	20/8/98	3/9/98	Returned	14/10/98	75
RT 229	6/8/98	Outbred	VIAS	*in vitro*	22	2M 15/5; 1-9(KO)	26/8/98	9/9/98	Returned	16/9/98	41
Minipig 2027	8/8/98	Minipig	MUMPF	*in vitro*	28	2M 15/5; 8-1(KO)	28/8/98	11/9/98	Returned	28/8/98	
RT 230	13/8/98	Outbred	VIAS	*in vitro*	40	2M 15/5; 5-7(KO)	2/9/98	16/9/98	Returned	23/9/98	41
RT231	14/8/98	Outbred	VIAS	*in vitro*	21	2M 15/5; 5-7(KO)	3/9/98	17/9/98	Returned	24/9/98	41

Pig ID	Embryo Transfer	Recipient Type	Location	Cytoplast Source	No. of NT embryos	NT cell line (minipig)	Return to Oestrus	Ultra-sound (35 days)	Status	Date of Return	Preg. Detect. (Days)
Minipig 1035	15/8/98	Minipig	MUMPF	*in vitro*	16	2M 15/5; 5-7(KO)	4/9/98	18/9/98	Returned	4/9/98	
RT 236	2/9/98	Outbred	VIAS	*in vivo*	25	1G5 P4 (WT)	22/9/98	6/10/98	Returned	23/9/98	
RT 237	3/9/98	Outbred	VIAS	*in vivo*	31	2M 15/5; 8-1(KO)	23/9/98	7/10/98	Not pregnant	14/10/98	Sacrificed
RT 238	4/8/98	Outbred	VIAS	*in vivo*	35	2M 15/5; 8-1(KO)	24/9/98	8/10/98	Pregnant		42
RT 239	17/9/98	Outbred	VIAS	*in vitro*	28	1G5 P4 (WT)	7/10/98	21/10/98	Pregnant		29
RT 240	18/9/98	Outbred	VIAS	*in vitro*	26	2M 15/5; 1-9(KO)	8/10/98	22/10/98	Returned	8/10/98	
RT 241	18/9/98	Outbred	VIAS	*in vitro*	27	2M 15/5; 1-9(KO)	8/10/98	22/10/98	Pregnant		28
RT 242	24/9/98	Outbred	VIAS	*in vitro*	20	1G5 P4 (WT)	14/10/98	29/10/98	Pregnant		22
RT 243	25/9/98	Outbred	VIAS	*in vitro*	21	2M 15/5; 1-9(KO)	15/10/98	29/10/98	Pregnant		21
RT 244	1/10/98	Outbred	VIAS	*in vitro*	18	2M 15/5; 1-9(KO)	21/10/98	4/11/98	NA		

Pig ID	Embryo Transfer	Recipient Type	Location	Cytoplast Source	No. of NT embryos	NT cell line (minipig)	Return to Oestrus	Ultra-sound (35 days)	Status	Date of Return	Preg. Detect. (Days)
RT245	2/10/98	Outbred	VIAS	*in vitro*	25	2M 15/5; 1-9(KO)	22/10/98	5/11/98	NA		

"WT" : wild type nuclei donor

"KC" : modified nuclei donor

Only reconstituted NT porcine embryos have been transferred to recipients.

While a published nuclear transfer approach has produced live offspring in cattle (Cibelli et al., 1998) our invention provides a new way of producing nuclear transfer bovine embryos (see Table 6).

TABLE 6

***In Vitro* Cattle Nuclear Transfer**

Procedure	Enucleation (%)	Fusion (%)	Development (%)	
			2/4-cell	16/32 cell
Activation - Enucleation - 6 hours - Fusion	41/55 (75)	37/41 (90)	10/37	4/37 (11)
Enucleation - 6 hours - Activation/Fusion Combined	45/50 (90)	30/45 (67)	5/30 (17)	2/30 (7)
Parthogenetic Activation			11/25 (44)	8/25 (32)

Karyoplast source

The technique developed for nuclear transfer has broad application to using donor nuclei other than foetal and embryonic cell lines. Reconstituted nuclear transfer porcine embryos were produced from transfer of adult pig fibroblast nuclei (see Table 7 and Example 2).

TABLE 7

Somatic Cell (Adult Cell) Nuclear Transfer in the Pig

Cell type	Procedure	Development to:	
		4 to 8 cell stage (%)	16 to 32 cell stage (%)
Adult Porcine Fibroblast	Activation - Enucleation - 6 hours fusion	25/45 (55)	16/45 (33)

Universal donor

The nuclear transfer technique was also assessed as to its suitability of the *in vitro* matured oocyte to service a universal donor.

Human fetal fibroblast were used as donor nuclei and fused to activated enucleated porcine oocytes. This demonstrated the utility of the nuclear transfer technique to provide development of nuclei to different species (see Table 8).

TABLE 8

Karyoplast Source (Human) for porcine Nuclear Transfer

Cell type	Procedure	Development to 16 to 32 cell stage (%)
Human Fetal Fibroblast	Activation - Enucleation - 6 hours - Fusion	2/7 (28)

Serial nuclear transfer

Alternatively, the activated reconstituted oocytes may be allowed to undergo one round of karyokinesis. The resulting nuclei can then be used for an additional round of nuclear transfer. This second round of nuclear transfer, or serial nuclear transfer, is postulated to improve the developmental capacity of the donor nuclei. Although the mechanism is unknown, re-exposure of the donor nuclei to molecular components in the cytoplasm of the cytoplast may assist in chromatin remodelling that is essential for nuclear reprogramming (Di Berardino 1987; Kwon and Kono 1996). To increase the number of nuclei available for serial nuclear transfer from one to two, the activated reconstituted cytoplasts were cultured in media with cytochalasin B (Sigma Cell Culture, Sigma-Aldrich Pty. Ltd., 5 bagel) at 39°C in 5%O CO_2 for 6 hours (Kwon and Kono 1996). Following activation the donor nucleus re-entered the cell cycle and completed karyokinesis. The addition of cytochalasin B (Sigma Cell Culture, Sigma-Aldrich Pty. Ltd.) to the media inhibited the extrusion of the second PB resulting in the formation of two nuclei. Karyoplasts were prepared from both nuclei and consequently used for a second round of nuclear transfer by placing the karyoplast into the PV space of the cytoplast. The cytoplast in the second round of nuclear transfer was usually a zonae pellucida intact, enucleated PN stage fertilised oocyte (see Example 1). Fusion was induced in a similar manner to that used for the first round of nuclear transfer. Activation was not required.

Embryo multiplication

To increase the number of embryos available for transfer following nuclear transfer, and therefore increase pregnancy rate, nuclear transfer may be performed using karyoplasts from 4-cell nuclear transfer embryos. In this example, the nuclear transfer procedure was used as a means of embryo multiplication. As cytoplasts reconstituted with karyoplasts at the same stage of the cell cycle demonstrate a higher developmental capacity (Cheong et al 1993; Kwon and Kono 1996), in this example metaphase arrested karyoplasts were fused to cytoplasts prepared from enucleated metaphase (MII) oocytes (see Example 1) using electrofusion and activation parameters described above. It was also

possible to prepare karyoplasts from nuclei in the late G2 stage of the cell cycle (see Example 2). Nuclei within these karyoplasts were allowed to progress through the cell cycle to mitosis or M phase, prior to fusion but were prevented from further progression through the cell cycle by incubation in nocodazole (Sigma Cell Culture, Sigma-Aldrich Pty. Ltd., 1 µg/ml).

EXAMPLE 8

Isolation of porcine somatic cell lines for nuclear transfer

Isolation

Nine foetuses were collected on day 28 of pregnancy from slaughtered DD miniature pig #12510 (G 5).

Cell lines from four tissue types have successfully been isolated from each foetus:

- pig embryonic lung (PEL) fibroblasts
- pig embryonic mesenchymal (PEM) fibroblasts
- pig embryonic heart (PEH) fibroblasts
- pig embryonic kidney (PEK) fibroblasts.

Propagation

Examples of all four cell types have been cultured for 9 passages (25 days). The results indicate that the growth curve (cumulative increase in cell number) and proliferative potential (growth ratio) of these cell lines is similar to that observed in previously isolated cell lines from commercial pig foetus.

Sex Determination

The sex of the isolated fibroblast cell lines was determined by PCR (Pomp et al., 1995).

Transfection efficiency

Electroporation is the method of choice for introduction of exogenous DNA into a fibroblast cell lines. However, other methods (e.g. lipofection) may also be used.

Electroporation parameters for each cell type have to date been optimised using control vector supplied by Peter Mountford. The results of these experiments were validated using the BTI construct (pGallaway). Other targeting constructs will be evaluated as they become available

Elimination of α1,3-galactosyltransferase activity in porcine embryonic fibroblasts

Porcine embryonic mesenchymal tissue derived fibroblasts were used to confirm the feasibility of elimination of α1,3-galactosyltransferase activity. The cell line, PEM, was transferred by electroporation with the pGallaway targeting bector linearized at a unique Xho I restriction site as described in Example 3. 1×10^6 PEM cells were suspended in 0.4 ml of HeBS (20 mM HEPES- NaOH pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose) containing 0.5 mM linearized pGallaway.

The cell suspension was placed in a 4 mm gap electroporation cuvette and kept at room temperature for 10 min. A 270 V, 960 µF charge was then applied with a Gene Pulser apparatus (Bio-Rad Laboratories).

After 10 min incubation at room temperature the cell suspension was transferred to 10 ml of culture medium and plated onto 10 cm Petri dish. The cells were re-fed culture medium containing 400 µg/ml Geneticin (Life Technologies) and selected for 8 - 14 days.

Following electroporation and selection with Geneticin aliquots of the stable transfectants were analysed by RT-PCR as described in Example 3. Two sets of primers were used:

1) wild type (primers F238 and R545) to verify that cDNA was transcribed successfully;

2) mutant (primers F238 and RD2) to identify the cells producing the mutant transcript expected from the homologous recombination of pGallaway and the endogenous α1,3-galactosyltransferase locus.

Initial analysis of a pool of 11 Geneticin resistant colonies resulted in detection of the expectant mutant template. The results of this analysis is shown in Figure 13A. Ethidium bromide staining of PCR products generated from cDNA with the wild type primers F238 and R545, and with the mutant primers F238 and RD2 are shown in panel A. Both PCR fragments have the same size. However, cleavage treatment with Ase I yields two fragments of 272 bp and 24 bp in the mutant PCR product but no cleavage in the wild type PCR product (Figure 13B).

The above results show that at least 1 out of 11 Geneticin resistant clones have mutant transcript expected from homologous recombination event. All 11 individual colonies have been stored in liquid N_2.

EXAMPLE 9

Culture of Embryos

Reconstituted oocytes were cultured in 20 µl drops of medium overlaid with paraffin oil (BDH Laboratory Supplies, UK or Sigma Chemical Company, Sigma-Aldrich Pty. Ltd.) under 5% CO2 and reduced O2 (5-7%) atmosphere at 39° C. G1 medium was used for the first 48 hr. followed by G2 medium. The combination of G1 and G2 medium and the reduced O2 greatly improved the quality of the early embryo and increased the number of cells that differentiated into inner cell mass cells.

Culture Media

Porcine embryos were incubated in a number of different types of culture

media. Two short term culture media were used for embryo collection, manipulation and embryo transfer:

1) Hepes (Sigma Cell Culture, Sigma-Aldrich Pty. Ltd. or Gibco BRL, Life Technologies inc.) buffered G1 medium with 4 mg/ml BSA (Miles Pentex or Gibco BRL, Life Technologies inc.)

2) Hepes (Sigma Cell Culture, Sigma-Aldrich Pty. Ltd. or Gibco BRL, Life Technologies inc.) buffered MEM with 5 mg/ml BSA (Miles Pentex or Gibco BRL, Life Technologies inc.). The following long term culture media were used:

1) Whittens medium with 15 mg/ml BSA (Miles Pentex or Gibco BRL, Life Technologies inc.)

2) G1/G2 medium with 4 mg/ml BSA (Miles Pentex or Gibco BRL, Life Technologies inc.).

The embryos were cultured in micro drops of medium overlaid with mineral oil (BDH Laboratory Supplies, UK or Sigma Chemical Company, Sigma-Aldrich Pty. Ltd.), in 5%CO_2: 5%O_2: 90%N_2 atmosphere, at 38.5°C. The embryos were cultured for up to 7 days before embryo transfer to suitable recipient female.

The chemical composition of G1 and G2 media is shown below. H-G1 is a hepes added version of G1 medium. All chemicals are Sigma, except for BSA (Gibco or Miles Pentex).

G1 from concentrated stocks

Each reagent was added to MQ H_2O as listed below, filtered and stored at 4°C.

Stock	10 ml	50 ml
A_{G1}	1.0 ml	5.0 ml
B	1.0 ml	5.0 ml
C_{G1}	01 ml	0.5 ml
D	0.1 ml	0.5 ml
G	0.1 ml	0.5 ml
T	0.1 ml	0.5 ml
ED	01 ml	0.5 ml
Non-Essential AA (x100)	0.1 ml	0.5 ml
H_2O	7.4 ml	37.0 ml
BSA	30 mg	150 mg

H-G1 from concentrated stocks

Each reagent was added to MQ H_2O as listed below, filtered and stored at 4°C.

Stock	10 ml	20 ml	50 ml
A_{G1}	1.0 ml	2.0 ml	5.0 ml
B	0.16 ml	0.32 ml	0.8 ml
C_{G1}	0.1 ml	0.2 ml	0.5 ml
D	0.1 ml	0.2 ml	0.5 ml
G	0.1 ml	0.2 ml	0.5 ml
T	0.1 ml	0.2 ml	0.5 ml
ED	0.1 ml	0.2 ml	0.5 ml
Non-Essential AA (X100)	0.1 ml	0.2 ml	0.5 ml
HEPES	0.84 ml	1.68 ml	4.2 ml
H_2O	7.4 ml	14.8 ml	37.0 ml
BSA	30 mg	60 mg	150 mg

G1 & H-G1 PREPARATION:

Preparation of Concentrated Stocks:

Concentrated stocks were prepared by dissolving components in sterile MQ H2O. Each stock was filtered and stored at 4°C for 3 months (Stock A_{G2}), 1 month (Stock D) or one week (Stocks B. D_{G2} and G).

Stock A_{G1}	Component	mM	g/100ml
(x10 concentration)	NaC1	85.16	5.265
	KC1	5.5	0.410
	$NaH_2PO_4.2H_2O$	0 5	0.078
	$MgSO_4\ 7H_2O$	1.0	0.246
	Glucose	0.5	0.091
	Penicillin	-	0.06
	Streptomycin	-	0.05
	NaLactate (50% syrup)	12.40	2.316

Stock B	Component	mM	g/100ml
(x 10:concentration	$NaHCO_3$	25.0	2.101
	Phenol Red	-	0.01[a]

Stock C_{G1}	Component	mM	g/10ml
(x 100 concentration)	NaPyruvate	0.32	0.0352

Stock D	Component	mM	g/10ml
(x 100 concentration)	$CaCl_2.2H_2O$	1.8	0.265

Stock G	Component	mM	g/10ml
(x 100 concentration)	Glutamine	1.0	0.146

Stock T	Component	mM	g/10ml
(x 100 concentration)	Taurine	0.1	0.0125

Stock ED	Component	mM	g/10ml
(x 100 concentration)	EDTA	0.1	0.029[b]

Stock HEPES	Component	mM	g/10ml
(x 100 concentration)	HEPES	20	5.958[c]
	Phenol Red	-	0.01[a]

[a] Usually only 0.0019 added

[b] EDTA must be dissolved in 220 µl 1 M NaOH prior to adding H_2O

[c] Adjust to pH 7.4 with NaOH dissolved in media. use pH strips as an initial indicator then confirm with pH meter.

G2 PREPARATION:

Preparation of Concentrated Stocks:

Concentrated stocks were prepared by dissolving components in sterile MQ H_2O. Each stock was filtered and stored at 4°C for 3 months (Stock A_{G2}), 1 month (Stock D) or one week (Stocks B. D_{G2} and G).

Stock A_{G1}	Component	mM	g/100ml
(x10 concentration)	NaCl	85 16	5.265
	KCl	5.5	0.410
	$NaH_2PO_4.2H_2O$	0.5	0.078
	$MgSO_4$ $7H_2O$	1.0	0.246
	Glucose	3.15	0.568
	Penicillin	-	0.06
	Streptomycin	-	0.05
	NaLactate (50% syrup)	11.74	2.193

Stock B	Component	mM	g/100ml
(x 10 concentration)	$NaHCO_3$	25.0	2.101
	Phenol Red		0 01[a]

Stock C_{G2}	Component	mM	g/10ml
(x 100 concentration)	NaPyruvate	O 10	O 011

Stock D	Component	mM	g/10ml
(x 100 concentration)	$CaCl_2.2H_2O$	1.8	0.265

Stock G	Component	mM	g/10ml
(x 100 concentration)	Glutamine	1.0	0.146

[a] Usually only 0.001g added

G2 from concentrated stocks

Each reagent was added to MQH_2O as listed below. The pH was measured and adjusted to pH 7.4 with NaOH (approx 50 µl 1M NaOH/10ml). The medium was filtered and stored at 4°C. Osmolarity should be 269 mOsm.

Stock	10 ml	50 ml
A_{G2}	1.0 ml	5.0 ml
B	1.0 ml	5.0 ml
CG2	0.1 ml	0.5 ml
D	0.1 ml	0.5 ml
G	0.1 ml	0.5 ml
Non-Essential AA (x100)	0.1 ml	0.5 ml
Essential AA (x50)	0.2 ml	0.5 ml
H_2O	7.4 ml	37.0 ml
BSA	30 mg	150 mg

EXAMPLE 10

Generation of transgenic Offspring by Nuclear Transfer

Viable offspring can be generated from transfer of embryonic or somatic cell nuclei to enucleated oocytes. Modification of this technology to include transgenic donor nuclei allows the generation of transgenic animals.

To demonstrate the generation of transgenic animals from nuclear transfer we have developed a novel approach using a mouse model. This approach involves the use of a unique transgenic mouse that had a nuclear localised lacZ-neoR expression as a result of random gene-trap integration of pGTIRES-βgeopA into what appears to be an ubiquitously expressed gene. As a result all cells from this mouse, including embryonic blastomeres, demonstrate distinctive blue nuclei when treated with the X-gal substrate. In this example, karyoplasts were prepared from embryonic blastomeres of this mouse and used in

nuclear transfer. Blastocysts that developed following *in vitro* culture of the reconstituted oocyte, were either treated with X-gal or transferred to a recipient female. All nuclei in the X-gal treated blastocysts displayed distinctively blue staining (Figure 12.1). Tissue samples from pups born following blastocyst transfer also displayed distinctively blue staining (Figure 12.2 nuclear transfer pups with litter mates and

Figure 12.3 X-gal stained tissues (tail tips) from nuclear transfer pups and litter mates). As this staining pattern is characteristic of the transgenic mouse from which the donor nuclei were isolated, this novel approach clearly confirmed that transgenic offspring can be generated from transgenic karyoplasts (nuclei) without loss of transgene expression.

EXAMPLE 11

MHC (SLA) Miniature Swine

A unique pig strain was used in the nuclear transfer program. The National Institute of Health (MHC/SLA) miniature swine is an inbred strain of swine with a genetically defined major histocompatibility complex. A breeding program was commenced in 1972 to develop a strain of inbred (MHC/SLA) miniature swine to increase homozygosity at loci associated with the swine lymphocyte antigen complex (Sachs et al., 1976).

Specific antibodies for each of the MHC (SLA) miniature pig strains have been isolated. Use of these strain specific antibodies allows unequivocal detection of contribution of karyoplast nuclei to the development of any pig produced by nuclear transfer.

EXAMPLE 12

Embryo Transfer in Outbred and MHC (SLA) Miniature swine.

Oestrous Synchronization

Several methods were developed to achieve synchronisation of both outbred and MHC (SLA) miniature swine. Selection of one particular method depends on the age and size of the animal, and on available facilities.

Method 1: Corpus luteum regression following Prostaglandin treatment

Recipient swine, 25 - 40 days pregnant (as confirmed by ultrasound or non return to estrus) were selected for embryo transfer. The following procedure was used to induce synchronisation:

Day 1 (am) Intramuscular injection with 4 ml of Juramate (Cloprostenol, Pitman-Moore Australia Ltd.) or
Prostaglandin F2α analogue.

Day 2 (am) intramuscular injection with 2 ml of Juramate (Cloprostenol, Pitman-Moore Australia Ltd.) or
Prostaglandin F2α analogue followed by 500 IU PMSG (Folligon, Intervet (Aust) Pty. Ltd. or Pregnecol, Horizon Technology Pty. Ltd.)

Day 5 (am) Intramuscular injection with 500 IU of hCG (Chorulon, Intervet (Aust) Pty. Ltd.) .

Day 6 Detection of estrus (standing heat)

Day 7 One cell embryo transfer.

Day 11 Blastocyst transfer.

Method 2: Natural cycling

Unstimulated, natural cycling recipient pigs were selected for embryo transfer. One cell embryos were transferred 24 hours after detection of estrus. Blastocysts were transferred 5 days after detection of estrus.

Method 3: Extended Progesterone treatment.

Natural cycling pigs were treated with Altrenogest (Regumate. Roussel-Ulcaf, Paris, France) for 14-18 days (15-20 mg/animal/day) to synchronise estrus cycles. Twenty four hours after removal of Regumate (Altrenogest, Hoechst-Roussel) swine were injected with 500 IU of PMSG (Folligon, Intervet (Aust) Pty. Ltd. or Pregnecol, Horizon Technology Pty. Ltd.) followed two days later injection of 500 IU of hCG (Chorulon, Intervet (Aust) Pty. Ltd.) .

Method 4: Synchronization of estrus of sows after lactation or weaning.

Recipients were synchronised for embryo transfer at weaning using the following procedure:

Day 1 Weaning (removal of piglets)

Day 2 (am) Intramuscular injection of 500 IU PMSG (Folligon. Intervet (Aust) Pty. Ltd. or Pregnecol, Horizon Technology Pty. Ltd.) Pty. Ltd.)

Day 5 (am) intramuscular injection of 500 IU hcG (Chorulon. Intervet (Aust)

Day 6 Detection of estrus (standing heat)

Day 7 One cell embryo transfer.

Day 11 Blastocyst transfer.

Surgery Standard surgical procedures were used. Following induction of

anaesthesia with Thiopentone, surgical anaesthesia was maintained with either halothane (fluothane, Zeneca, ICI Australia Operations Pty. Ltd.) or Isofluorane (Forthane, Abbott Labs, UK) in oxygen. Reproductive tracts were exteriorised by midline laparotomy.

Oocytes or pronuclear stage embryos were collected through a modified teflon catheter inserted into the oviduct via the infundibullum. Embryos were collected by retrograde flushing with flushing medium (Dulbecco's phosphate buffered saline (Gibco BRL, Life Technologies Inc., NY or Trace Biosciences Pty. Ltd. [D-PBS] supplemented with 1% fetal calf serum (CSL Limited, Aus), calcium and magnesium).

Embryos were transferred in short term culture medium. One to four cell embryos were transferred to the oviduct of a recipient using a tom cat catheter and 1 ml syringe. Eight cell to blastocysl stage embryos were transferred to the uterus of the recipient using the same equipment.

Recipients were synchronised to the development stage of the embryos transferred. Improved pregnancy rates were achieved by asynchronous transfer. The estrous cycles of recipient female pigs were programmed 24 hours behind embryo development. Approximatoly 67% of recipients farrow following such embryo transfer.

Finally, it is to be understood that various alterations, modifications and/or additions may be made without departing from the spirit of the present invention as outlined herein.

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CLAIMS

1. A method of generating an animal embryo, which method includes providing
a donor nucleus, and
a recipient cell;
removing the nucleus for the recipient cell;
introducing the donor nucleus into the enucleated cell to produce a couplet; and
maintaining the couplet in a suitable medium for a period sufficient to allow the cell to recover a substantially normal shape.

2. A method according to Claim 1 further comprising an activation step

3. A method according to Claim 2 wherein the couplet is subsequently subjected to a cell fusion step.

4. A method according to Claim 72 wherein the couplet is subsequently subjected to a cell fusion/activation step.

5. A method according to Claims 2, 64 or 65 wherein the animal embryo is a murine, bovine, ovine or porcine embryo.

6. A method according to Claim 5 wherein the embryo is a porcine embryo.

7. A method according to Claim 6 where the donor is selected from the group consisting of embryonic, foetal and adult cells.

8. A method according to Claim 7 wherein the donor nucleus is from a porcine animal.

9. A method according to Claim 8 wherein the donor nucleus is from an MHC (SLA)-miniature swine.

10. A method according to Claims 2, 64 or 65 1 wherein the recipient cytoplasm is prepared from *in vivo* or *in vitro* produced oocytes.

11. A method according to Claim 10 wherein the oocytes are oocytes which have been subjected to an *in vitro* maturation step.

12. A method according to Claim 11 wherein the *in vitro* maturation is conducted in the presence of estrus cow serum.

13. A method according to Claim 1 wherein the couplet is maintained in a suitable medium for a period of approximately 3 to 8 hours.

14. A method according to Claim 13 wherein the couplet cytoplasm is maintained in a suitable medium for a period of approximately 4.5 to 6 hours.

15. A method according to Claim 1 wherein the donor nucleus is introduced into the enucleated cell substantially immediately after enucleation.

16. A method according to Claim 15 wherein the nucleus is removed from the activated cell via an incision site and the donor nucleus is introduced into the enucleated cell through the same incision site.

17. A method according to Claim 16 wherein the donor nucleus s introduced under the zona pellucida.

18. A method according to Claim 1 wherein the donor nucleus is genetically modified by random transgene integration or targeted transgene integration in an endogenous gene in the donor nucleus.

19. A method according to Claim 18 wherein the endogenous gene in the donor nucleus is modified by introducing into said donor nucleus a DNA construct including a nucleic acid sequence which is substantially isogenic to at least one or more portions of the endogenous gene and includes one or more mutations, such that there is homologous recombination between the construct and the endogenous gene.

20. A method according to Claim 19 wherein the animal nucleus is a porcine nucleus and the endogenous gene is the α1,3-galactosyltransferase gene.

21. A method according to Claim 20 wherein the DNA construct is a plasmid selected from the group consisting of pGallaway, pBertI and pBertII, or a functionally equivalent plasmid.

22. A method according to Claim 1 wherein the couplet is cultured *in vitro* to produce the transgenic animal embryo and then the transgenic animal

embryo is transferred to a surrogate for subsequent development into a transgenic animal.

23 An animal embryo produced by a method according to Claim 1.

24. A method of generating a transgenic animal embryo which method includes providing
a donor nucleus which has been genetically modified to eliminate an undesirable activity or to provide for a desirable activity, and a recipient cell;
transferring the donor nucleus to the recipient cell to produce a couplet; and
generating a transgenic animal embryo from said couplet.

25. A method according to Claim 24 wherein the transfer step includes
removing the nucleus from the recipient cell;
introducing the donor nucleus into the enucleated cell to produce a couplet; and
maintaining the couplet in a suitable medium for a period sufficient to allow the cell to recover a substantially normal shape.

26. A method according to Claim 25 including the preliminary step of subjecting the recipient cell to an activation step; and
subsequently removing the nucleus from the activated cell.

27. A method according to Claim 24 wherein the transgenic animal embryo is a porcine embryo.

28. A method according to Claim 27 wherein the donor is selected from the group consisting of embryonic, foetal and adult cells.

29. A method according to Claim 28 wherein the donor nucleus is from a MHC (SLA) miniature swine.

30. A method according to Claim 24 wherein the recipient cell is prepared by enucleation of *in vivo* or *in vitro* produced oocytes.

31. A method according to Claim 30 wherein the oocytes are oocytes which have been subjected to an *in vitro* maturation step.

32. A method according to Claim 31 wherein the *in vitro* maturation is conducted in the presence of estrus cow serum.

33. A method according to Claim 29 wherein the recipient cell is from a MHC (SLA) miniature swine.

34. A method according to Claim 26 wherein subsequent to the transfer step, the membrane fusion of the couplet is induced.

35. A method according to Claim 34 wherein the membrane fusion is induced by exposing the couplet electrical pulses or polyethylene glycol.

36. A method according to Claim 34 wherein the recipient cell is activated by exposure to an agent selected from the group consisting of ethanol, calcium ionophore and electrical stimulation.

37. A method according to Claims 24, 25 or 26 wherein the donor nucleus is from an embryo that is itself a product of nuclear transplantation.

38. A method according to Claim 37 wherein the donor nucleus is from an embryo that is a product of serial nuclear transplantation.

39. A method according to Claims 24, 25 or 26 wherein the donor nucleus is genetically modified by random transgene integration or targeted transgene integration in an endogenous gene in the donor nucleus.

40. A method according to Claim 39 wherein the endogenous gene in the donor nucleus is modified by introducing into said donor nucleus a DNA construct including a nucleic acid sequence which is substantially isogenic to at least one or more portions of the endogenous gene and includes one or more mutations such that there is homologous recombination between the construct and the endogenous gene.

41. A method according to Claim 24 wherein the undesirable activity is xenoantigenicity.

42. A method according to Claim 41 wherein the animal nucleus is a porcine nucleus and the endogenous gene is the α1,3-galactosyltransferase gene.

43. A method according to Claim 42 wherein the DNA construct is a plasmid selected from the group consisting of pGallaway, pBertI and pBertII or a functionally equivalent plasmid.

44. A method according to Claim 34 or claim 67 wherein the couplet is cultured *in vitro* to produce the transgenic animal embryo and then the transgenic animal embryo is transferred to a surrogate for subsequent development into a transgenic animal.

45. A transgenic animal embryo produced by a method according to Claims 24, 25 or 26.

46. A transgenic porcine embryo produced by a method according to Claims 24, 25 or 26.

47. A transgenic animal produced by a method according to Claim 38.

48. A transgenic animal according to Claim 40 wherein the expression of an endogenous gene has been deleted.

49. A transgenic animal descended from a transgenic animal according to claim 47.

50. A transgenic animal descended from transgenic animal according to claim 48.

51. An organ derived from a transgenic animal according to Claim 47.

52. Tissue derived from a transgenic animal according to claim 47.

53. Cells derived from a transgenic animal according to Claim 47.

54. A DNA construct for modifying an endogenous gene in an animal nucleus, said DNA construct including a nucleic acid sequence which is substantially isogenic to at least one or more portions of the endogenous gene and includes one or more mutations.

55. A DNA construct according to Claim 54 wherein the animal nucleus is a swine nucleus and the endogenous gene is• • 1,3-galactosyltransferase.

56. A DNA construct according to Claim 55 which is pGallaway or a functionally equivalent plasmid.

57. A DNA construct according to Claim 54 further including a selectable marker such that functional integration of said DNA construct into said endogenous gene results in expression of the selectable marker

58. A DNA construct according to Claim 57 wherein the animal nucleus is a swine nucleus and the endogenous gene is • 1,3-galactosyltransferase.

59. A DNA construct according to Claim 58 which is pBERT I or a functionally equivalent plasmid.

60. A DNA construct according to Claim 59 further including recombination sites flanking said selectable marker such that addition of a recombinase results in removal of the selectable marker by recombination at the recombination sites.

61. A DNA construct according to Claim 60 wherein the animal nucleus is a swine nucleus and the endogenous gene is • 1,3-galactosyltransferase.

62. A cell or cell line containing a DNA construct according to Claim 54.

63. A method according to Claim 25 wherein, subsequent to the transfer step, membrane fusion/activation of the couplet is induced.

64. A method of generating an animal embryo, which method includes providing
a donor nucleus, and
a recipient cell;
removing the nucleus from the recipient cell;
maintaining the enucleated cell in a suitable medium for a period sufficient to allow the cell to recover a substantially normal shape;
and transferring the donor nucleus to the enucleated cell by direct injection.

65. A method according to Claim 64, further comprising an activation step.

66. A method according to Claims 64 or 65 wherein the enucleated cell is maintained in a suitable medium for a period of approximately 3 to 8 hours.

67. A method according to Claims 64 or 65 wherein the donor nucleus has been genetically modified to eliminate an undesirable activity or to provide for a desirable activity.

68. A transgenic animal produced by a method according to Claims 64, 65 or 67.

69. A transgenic animal descended from a transgenic animal according to Claim 68.

70. The method according to Claim 65, wherein the activation step is after the direct injection.

71. The method according to Claim 65, wherein the activation step is prior to removing the nucleus from the recipient cell.

72. The method according to Claim 2, wherein the activation step is after the introduction of the donor nucleus into the enucleated cell.

73. The method according to Claim 2, wherein the activation step is prior to removing the nucleus from the recipient cell.

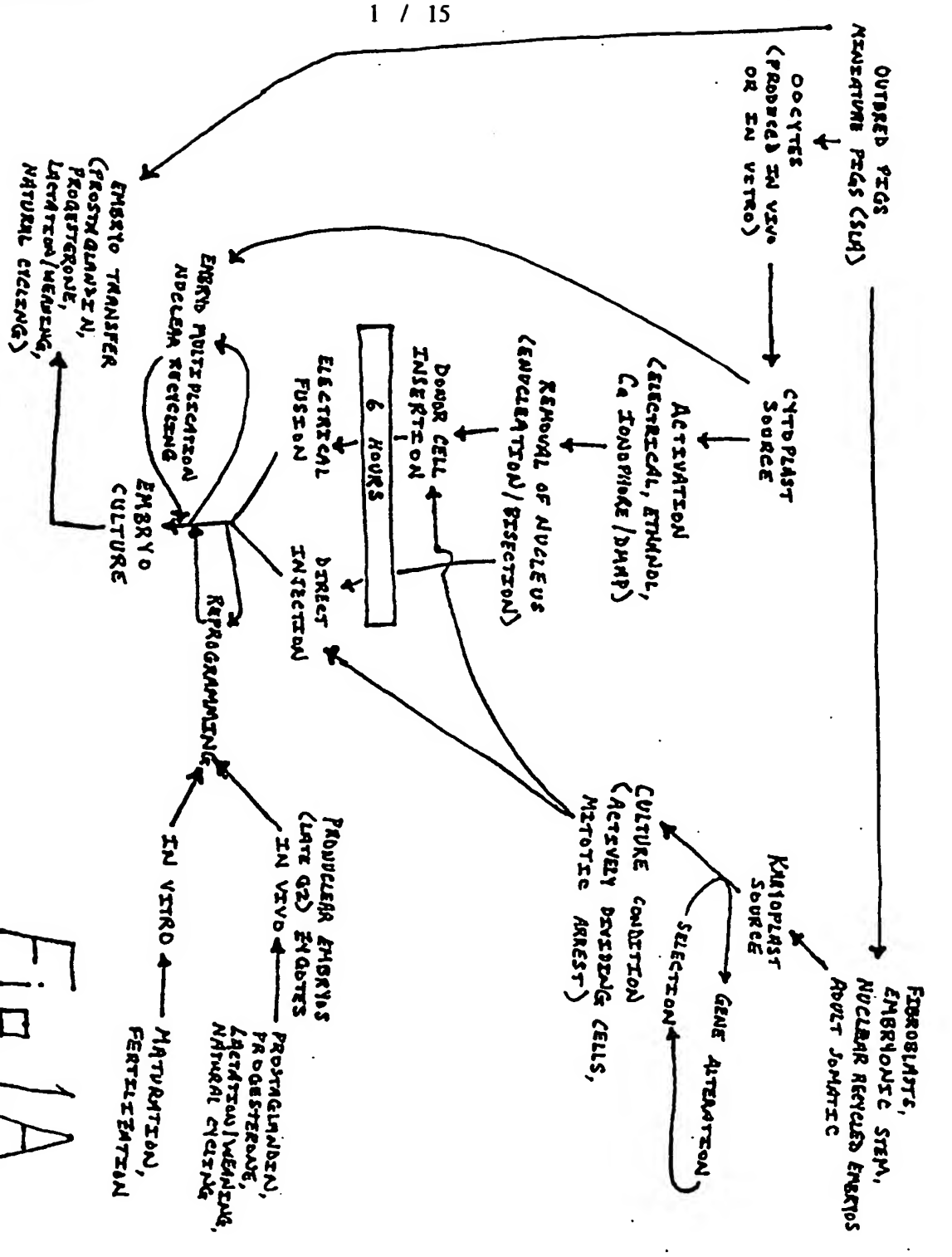

OUTBRED PIGS
NEGATIVE PIGS (SLA)
OOCYTES
(PRODUCED IN VIVO OR IN VITRO)
CYTOPLAST SOURCE
ACTIVATION
(ELECTRICAL, ETHANOL, Ca IONOPHORE / DMAP)
REMOVAL OF NUCLEUS
(ENUCLEATION / BISECTION)
DONOR CELL INSERTION
6 HOURS
ELECTRICAL FUSION
DIRECT INJECTION
EMBRYO FUSION AFTER NUCLEAR RECEIVING
EMBRYO CULTURE
EMBRYO TRANSFER
(PROSTAGLANDIN, PROGESTERONE, LACTATION/WEANING, NATURAL CYCLING)
REPROGRAMMING
IN VITRO
MATURATION, FERTILIZATION
PRONUCLEAR EMBRYOS (LATE G2) ZYGOTES
IN VIVO
PROSTAGLANDIN, PROGESTERONE, LACTATION/WEANING, NATURAL CYCLING
FIBROBLASTS, EMBRYONIC STEM, NUCLEAR RECIPIENT ADULT SOMATIC
KARIOPLAST SOURCE
GENE ALTERATION
SELECTION
CULTURE CONDITION (ACTIVELY DIVIDING CELLS, MITOTIC ARREST)

Fig. 1A

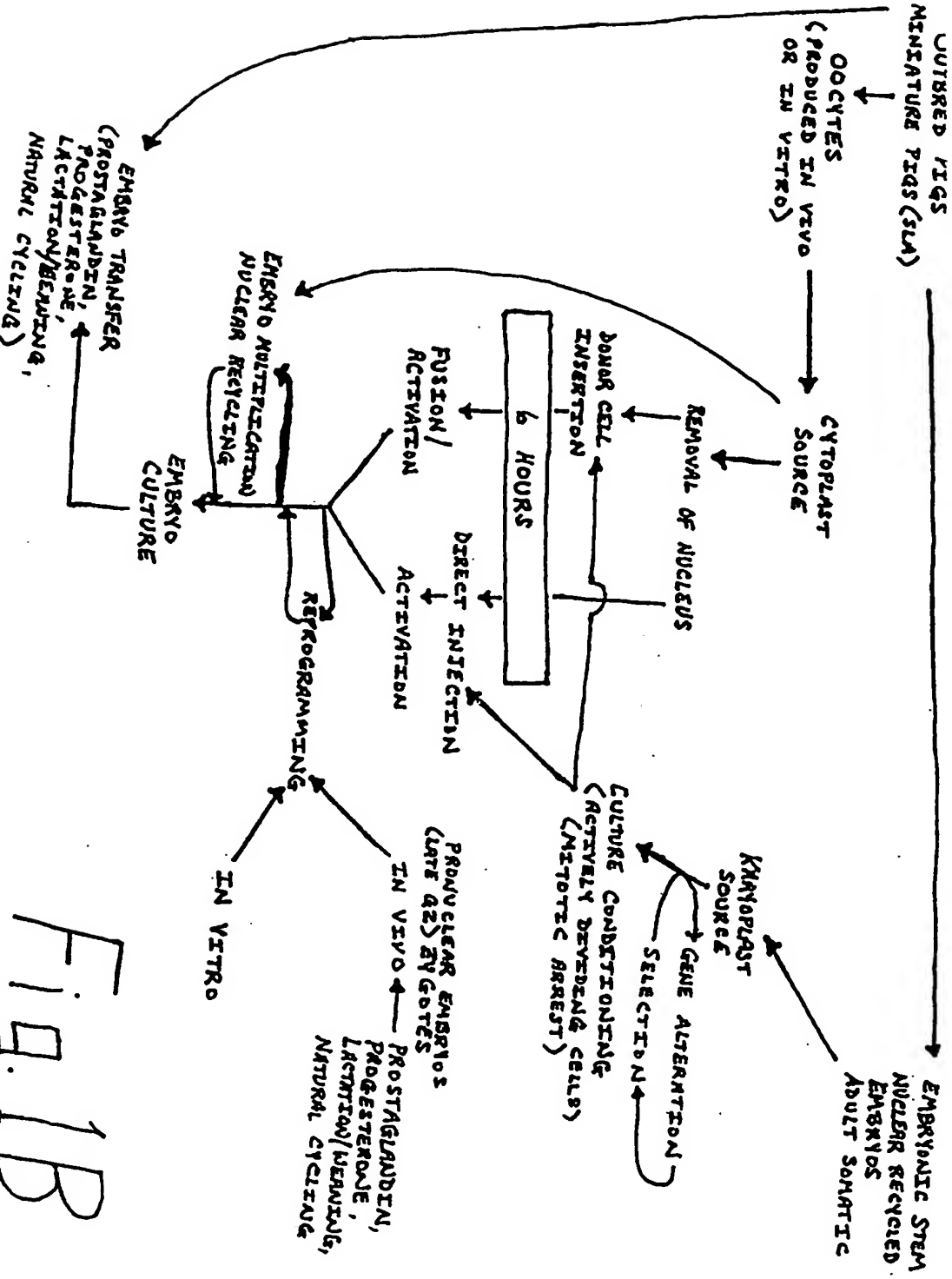

OUTBRED PIGS
MINIATURE PIGS (SLA)
OOCYTES
(PRODUCED IN VIVO
OR IN VITRO)
CYTOPLAST
SOURCE
REMOVAL OF NUCLEUS
DONOR CELL
INSERTION
6 HOURS
FUSION/
ACTIVATION
DIRECT INJECTION
ACTIVATION
REPROGRAMMING
EMBRYO MULTIPLICATION
NUCLEAR RECYCLING
EMBRYO
CULTURE
EMBRYO TRANSFER
(PROSTAGLANDIN,
PROGESTERONE,
LACTATION/WEANING,
NATURAL CYCLING)
IN VITRO
PRONUCLEAR EMBRYOS
(LATE G2) ZYGOTES
IN VIVO
PROSTAGLANDIN,
PROGESTERONE,
LACTATION/WEANING,
NATURAL CYCLING
CULTURE CONDITIONING
(ACTIVELY DIVIDING CELLS)
(MITOTIC ARREST)
KARYOPLAST
SOURCE
GENE ALTERATION
SELECTION
EMBRYONIC STEM
NUCLEAR RECYCLED
EMBRYOS
ADULT SOMATIC

Fig. 1B

pGallaway Construction

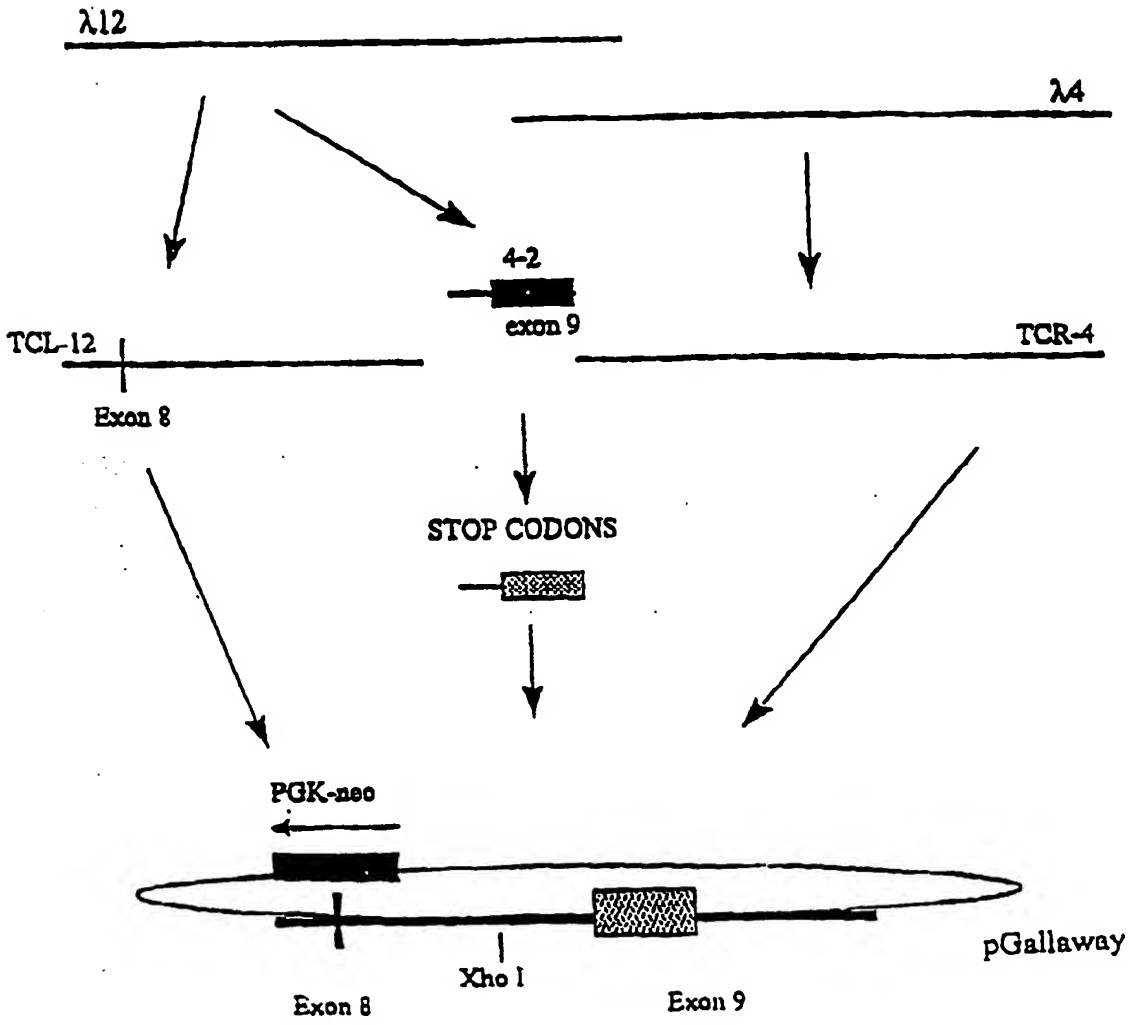
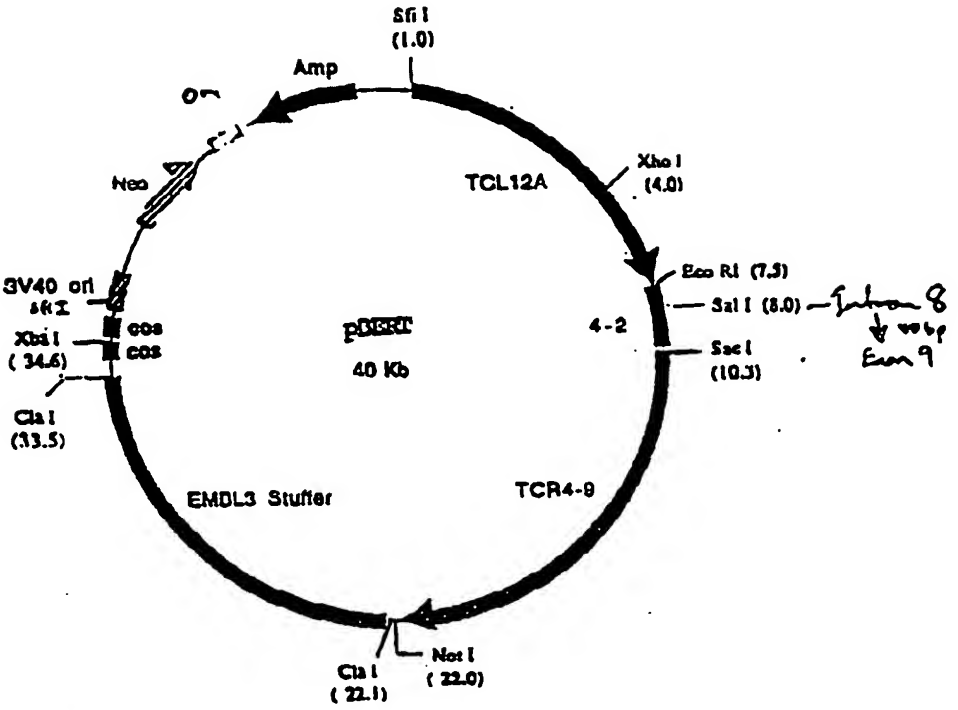


Figure 2

FIGURE 3

pBERT



Plasmid name: pBERT
Plasmid size: 40 Kb

Comments/References:

Map positions are approximate only

Vector backbone is Stratagene SuperCos (7.6-7.9 Kb; catalog # 251301) -- Eco RI/Not I

EMBL3 Stuffer is 11.4 Kb Cla I fragment (U02426)

4-2 is the 2.8 Kb Eco RI/Sac I fragment from lambda clone #12 and contains exon 9 coding region. The Eco RI fragment within 4-2 has been modified such that a unique Sal I site has been engineered into intron 8 and there are in-frame stop codons within the coding region of exon 9.

For use as an insertion vector, linearize with Sal I

For use as a replacement vector, digest with Sfi I/Not I. Note: there is an Sfi I site in Supercos.

TCL12A is from lambda clone #12 -- ~7.5 Kb Sac I/Eco RI fragment

TCR4-9 is from lambda clone #4 -- ~11.8 Kb Sac I/Not I fragment

This construct may be modified to delete the SV40 ori.

FIGURE 4

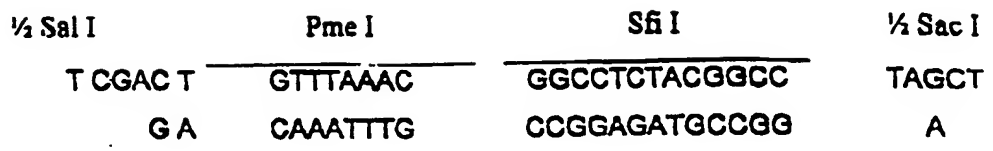
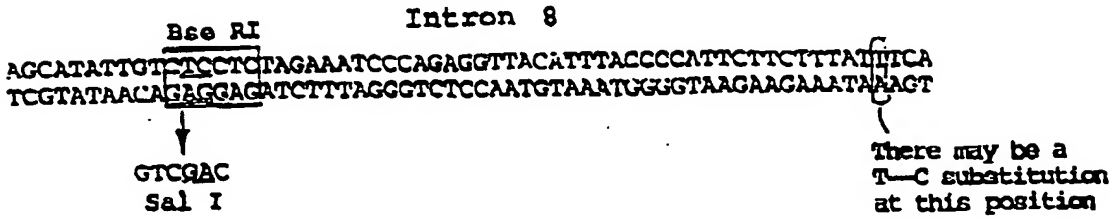
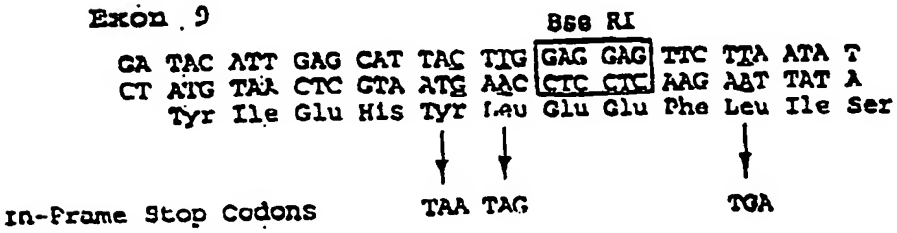

½ Sal I
Pme I
Sfi I
½ Sac I
T CGAC T
GTTTAAAC
GGCCTCTACGGCC
TAGCT
G A
CAAATTTG
CCGGAGATGCCGG
A

Figure 5 pBERT - Modifications to the 4-2 Fragment





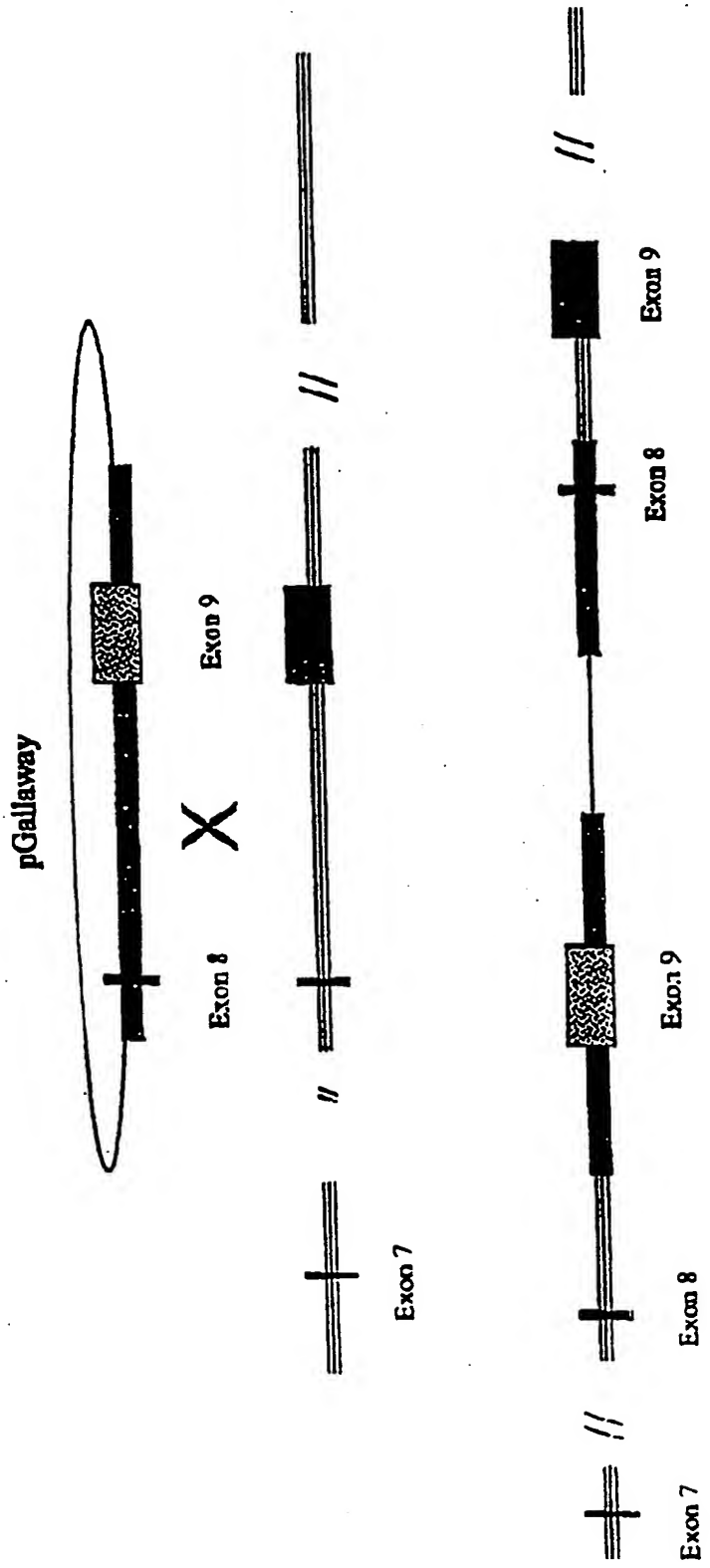


Figure 6

Detection of RT-PCR Homologous Recombination

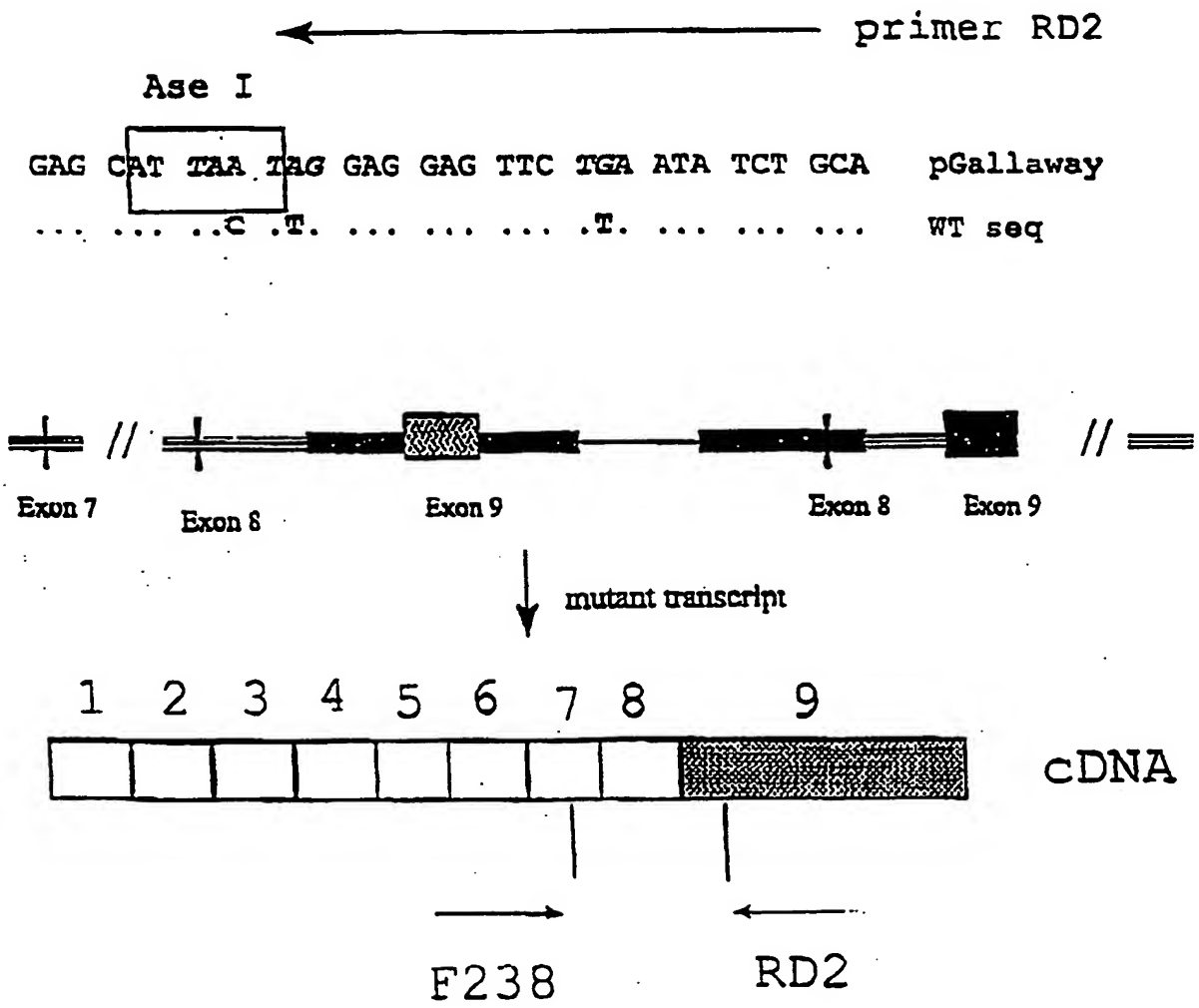
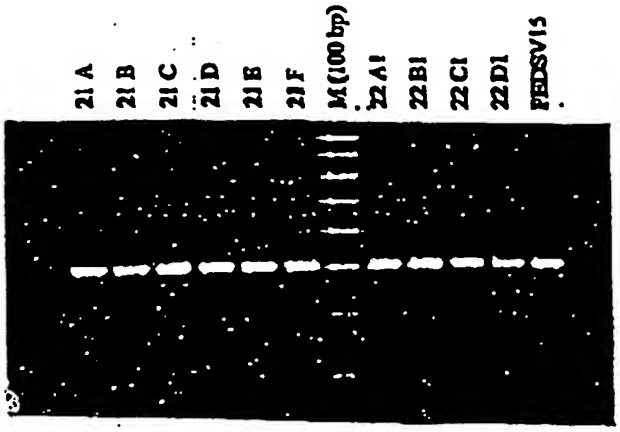


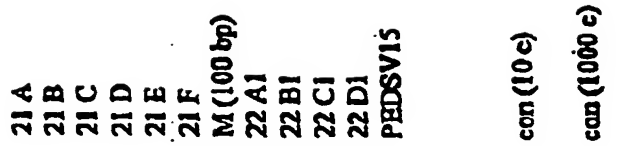
Figure 7

RT-PCR Analysis of Transfectant Pools



A F238/R545
WT Primers

B F238/RD2
Mutant Primers
+ RT



C F238/RD2
Mutant Primers
- RT

Figure 8

Ase I Digestion of Mutant PCR Products

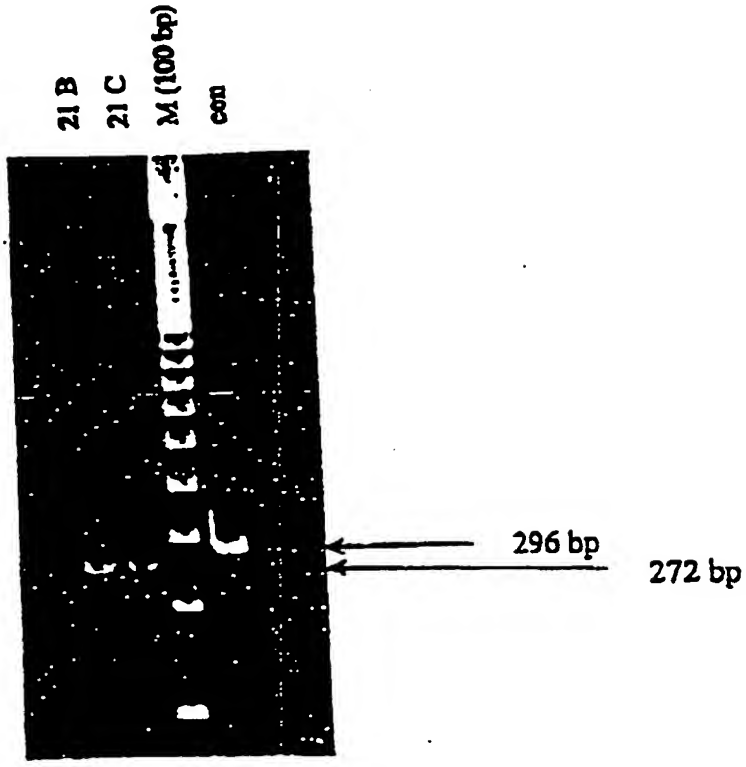
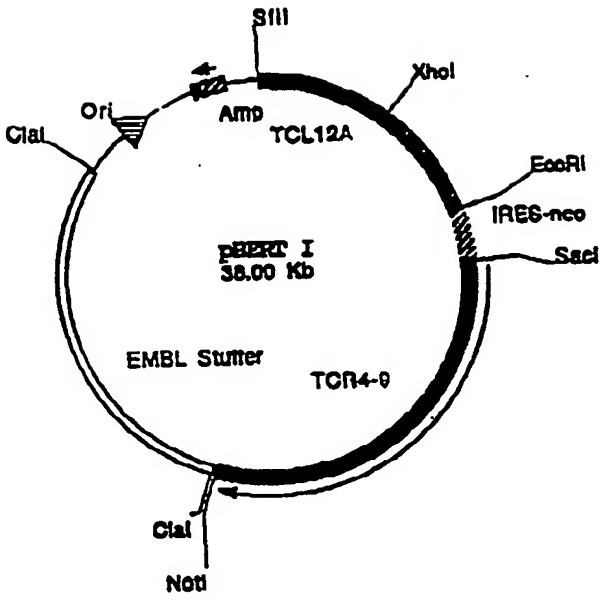


Figure 9

FIGURE 10


SfiI
XhoI
Ori
Amp
ClaI
TCL12A
EcoRI
IRES-neo
SacI
pBERT I
38.00 Kb
EMBL Stuffer
TCR4-9
ClaI
NotI

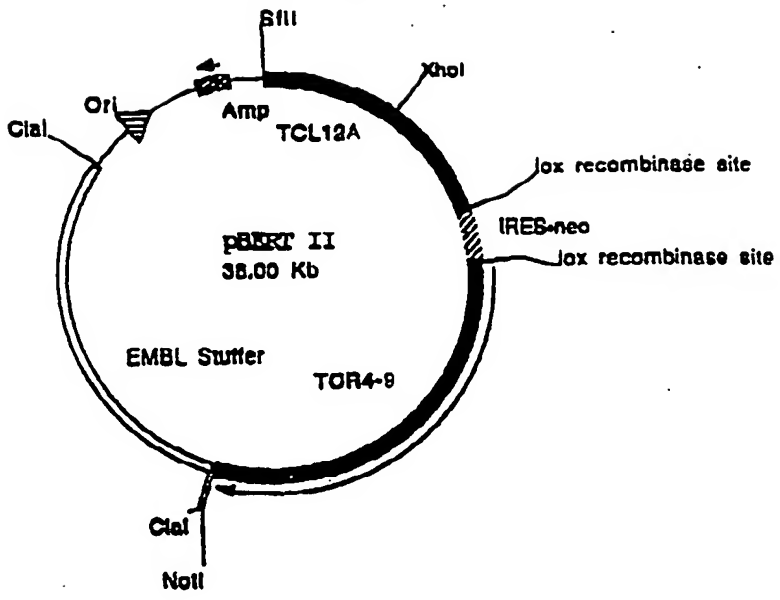

SfiI
XhoI
Ori
Amp
ClaI
TCL12A
lox recombinase site
IRES-neo
pBEKT II
38.00 Kb
lox recombinase site
EMBL Stuffer
TOR4-9
ClaI
NotI

FIGURE 11

Figure 12.1: X-gal stained hatching blastocysts (day 4 of culture) developed following nuclear transfer of karyoplasts derived from transgenic (pGTIRES-βgeopA) or wild type blastomeres.

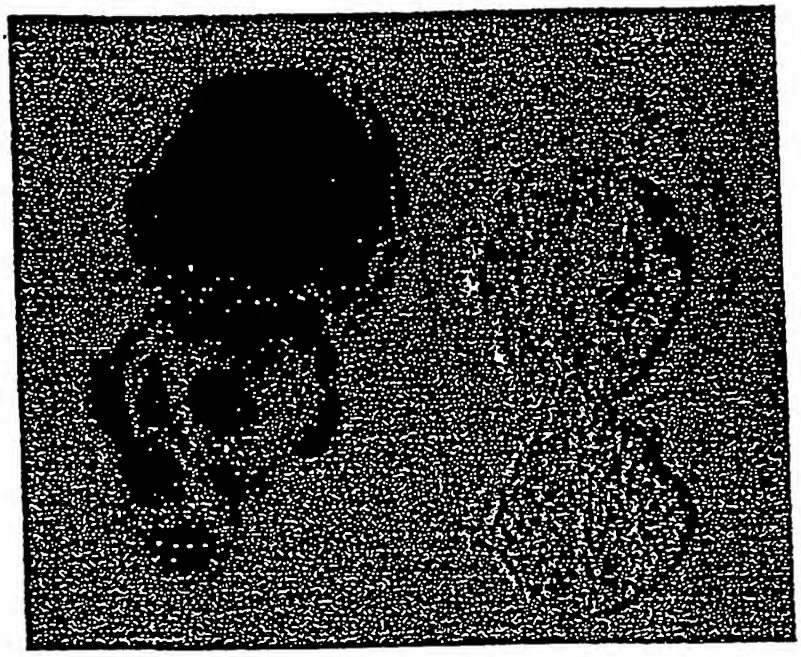


Figure 12.2: Transgenic nuclear transfer pups with non transgenic litter mates (19 days post partum).

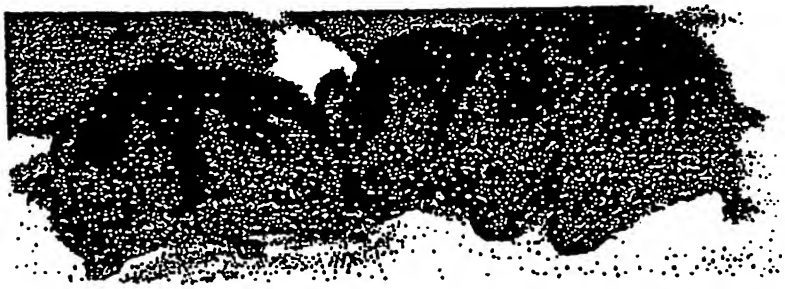
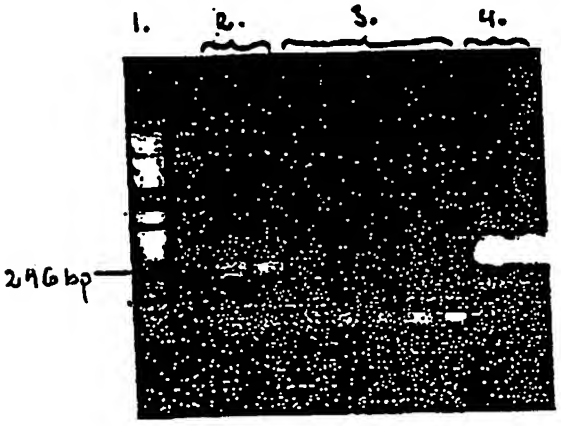


Figure 12.3: X-gal stained tail samples confirming generation of transgenic mice following nuclear transfer (three negatives and two positives).

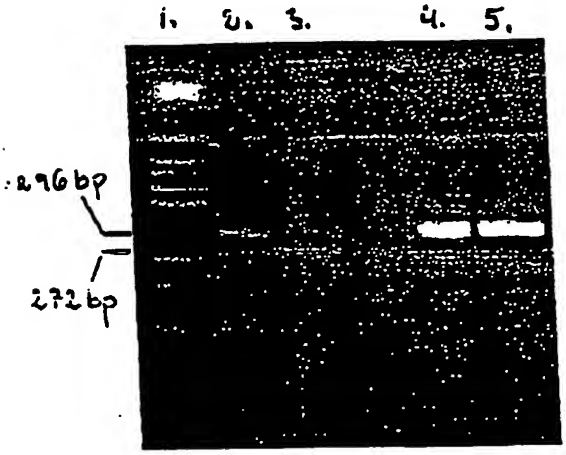
Figure 13 Elimination of α1,3 - galactosyltransferase Activity in Porcine Embryonic Fibroblasts.

A. RT-PCR analysis of Geneticin resistant pool
(two RNA preparations of the same pool were analysed)



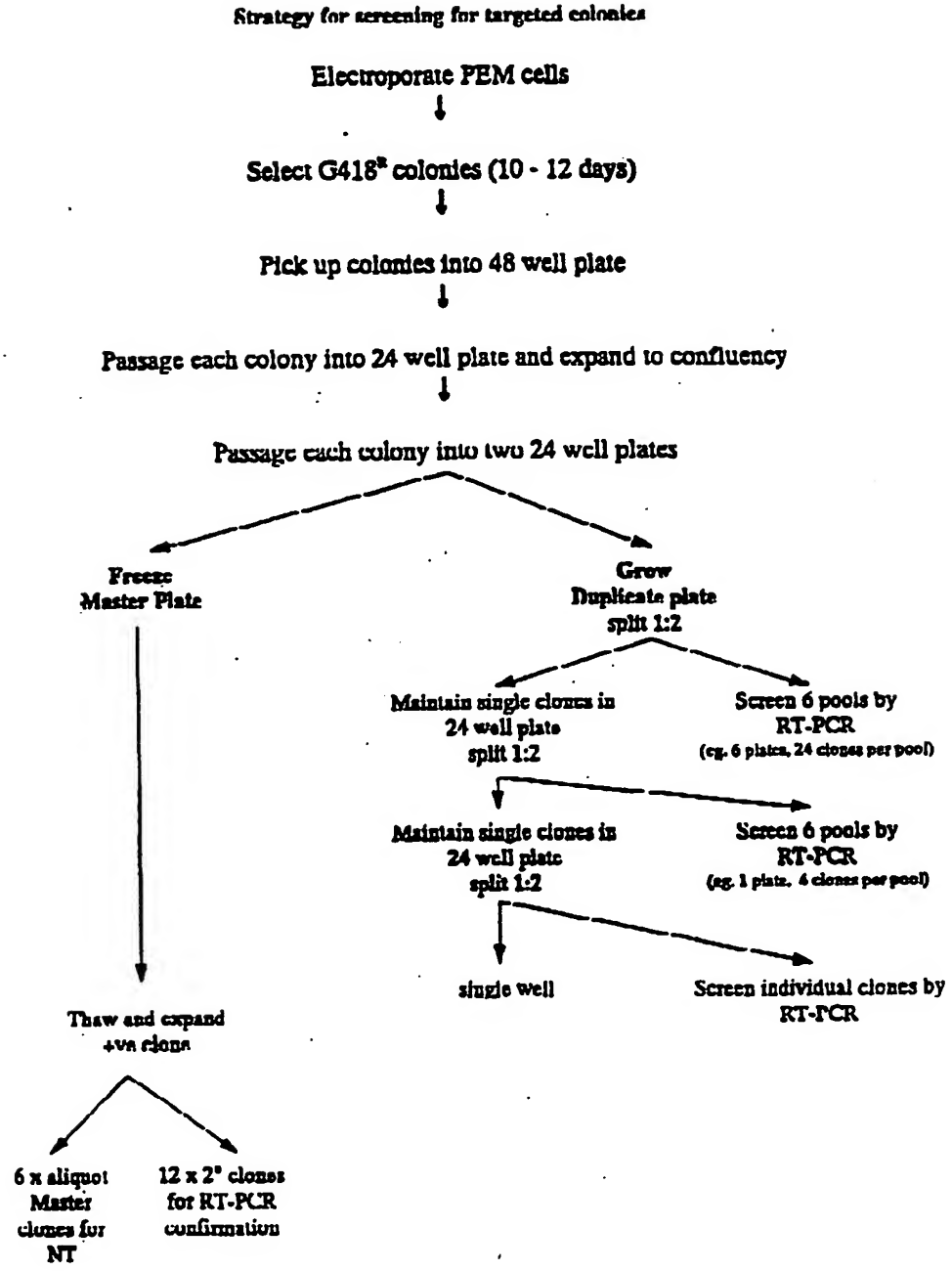
1: pGEM molecular marker
2. F238/RD mutant primers (MT)
3. negative controls
4. F238/R545 wild type primers (WT)

B. Ase I digestion of mutant PCR product



1. pGEM molecular marker
2. MT
3. MT/Ase I
4. WT
5. WT/Ase I

FIGURE 14


Strategy for screening for targeted colonies
Electroporate PEM cells
Select G418R colonies (10 - 12 days)
Pick up colonies into 48 well plate
Passage each colony into 24 well plate and expand to confluency
Passage each colony into two 24 well plates
Freeze Master Plate
Grow Duplicate plate split 1:2
Maintain single clones in 24 well plate split 1:2
Screen 6 pools by RT-PCR
(eg. 6 plates, 24 clones per pool)
Maintain single clones in 24 well plate split 1:2
Screen 6 pools by RT-PCR
(eg. 1 plate, 4 clones per pool)
single well
Screen individual clones by RT-PCR
Thaw and expand +ve clone
6 x aliquot Master clones for NT
12 x 2° clones for RT-PCR confirmation

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/22882

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A01K 67/027, C12N 5/10, C12N 15/87, C12N 9/10
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A01K, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5453366 A (MICHELE M. SIMS ET AL), 26 Sept 1995 (26.09.95), column 3, line 42 - line 64; column 7, line 63 - column 8, line 15	1-73
X	Int J. Dev. Biol., Volume 39, 1995, Lin Liu et al, "Nuclear remodelling an early development in cryopreserved, porcine primordial germ cells following nuclear transfer into in vitro-matured oocytes", page 639 - page 644, page 643, column 1, line 12 - line 38	1-73
X	Berl. Münch. Tierärztl. Wschr., Volume 105, 1992, Von Annette Clement-Sengewald et al, "Zur Embryoklonierung von Nutztieren", page 15 - page 21, figure 1	1-73

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
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"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 March 1999	12 04 99

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Authorized officer
PATRICK ANDERSSON

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/22882

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Biology of Reproduction, Volume 41, 1989, Randalls S. Prather et al, "Nuclear Transplantation in Early Pig Embryos" page 414 - page 418	1-73
X	WO 9707669 A1 (ROSLIN INSTITUTE), 6 March 1997 (06.03.97), page 27, line 8 - line 12, claims	1-73
X	WO 9517500 A1 (ABS GLOBAL, INC.), 29 June 1995 (29.06.95), page 26, line 15 - line 27; page 31, line 14 - line 25, claims	1-73
A	WO 9716064 A1 (THE MOUNT SINAI MEDICAL CENTER), 9 May 1997 (09.05.97), claims	20-21,24-63

INTERNATIONAL SEARCH REPORT

Information on patent family members

02/02/99

International application No.
PCT/US 98/22882

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		US 5096822 A	17/03/92
WO 9707669 A1	06/03/97	AU 6831096 A	19/03/97
		CA 2229568 A	06/03/97
		CZ 9800608 A	15/07/98
		EP 0849990 A	01/07/98
		GB 2318578 A	29/04/98
		GB 9517780 D	00/00/00
		GB 9802915 D	00/00/00
		GB 9824304 D	00/00/00
		IL 123298 D	00/00/00
		NO 980845 A	29/04/98
		PL 325331 A	20/07/98
WO 9517500 A1	29/06/95	AU 1595195 A	10/07/95
		EP 0739412 A	30/10/96
WO 9716064 A1	09/05/97	AU 7668696 A	22/05/97
		EP 0877549 A	18/11/98